R. N. H. KONINGS

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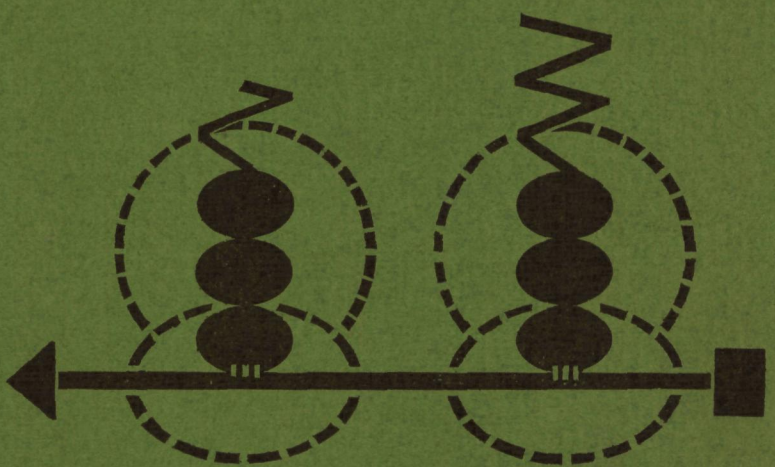

MESSENGER ACTIVITY IN RNA PREPARATIONS FROM CALF LENS AND RAT LIVER

PROMOTOR: PROF. DR. H. BLOEMENDAL

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MESSENGER ACTIVITY IN RNA PREPARATIONS FROM CALF LENS AND RAT LIVER

PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD VAN DOCTOR
IN DE WISKUNDE EN NATUURWETENSCHAPPEN AAN DE
KATHOLIEKE UNIVERSITEIT TE NIJMEGEN, OP GEZAG
VAN DE RECTOR MAGNIFICUS MR. S. F. L. BARON VAN WIJNBERGEN,
HOOGLERAAR IN DE FACULTEITEN DER RECHTSGELEERDHEID
EN DER SOCIALE WETENSCHAPPEN,
VOLGENS BESLUIT VAN DE SENAAT IN HET OPENBAAR TE
VERDEDIGEN OP DINSDAG 24 JUNI 1969
DES NAMIDDAGS TE 4 UUR

DOOR

RUDOLPH NICOLAAS HENDRIK KONINGS

GEBOREN TE HEER

1969
DRUKKERIJ GEBR. JANSSEN N.V. NIJMEGEN

...natural mRNA often seems to resemble the Scarlet Pimpernel:

'We seek him here, we seek him there,
those Frenchies seek him everywhere.
Is he in heaven? – Is he in hell?
That demmed, elusive Pimpernel?'

M. F. SINGER AND P. LEDER

Aan de nagedachtenis van mijn vader
Aan mijn moeder, Lize en Bastiaan

Het verschijnen van dit proefschrift biedt me een welkome gelegenheid mijn dank uit te spreken aan al diegenen die op enigerlei wijze tot het tot stand komen hiervan hebben bijgedragen.

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CONTENTS

INTRODUCTION

The central working hypothesis of molecular genetics states that DNA directs the synthesis of an RNA which in turn directs the synthesis of protein. This may be schematically indicated as follows:

$$\text{DNA} \rightarrow \text{RNA} \rightarrow \text{protein.}$$

The validity of this two-step process is now generally accepted, nevertheless the final proof would be an imitation of it in a cell-free system.

The second step of this process has been simulated in cell-free systems. In 1961 Nirenberg and Matthaei demonstrated for the first time that synthetic polynucleotides stimulate amino acid incorporation into protein in the cell-free system prepared from *E. coli*.

This spectacular result made it possible to decipher the genetic code (Nirenberg et al., 1963; Speyer et al., 1963; Söll et al., 1964). In addition the demonstration of the synthesis of specific proteins directed by synthetic and natural messenger RNA's in this and other cell-free systems, strongly suggest that the genetic code is universal. (Nathans et al., 1962; Fessenden et al., 1963a, 1963b; Clark et al., 1965; Nishimura et al., 1965; Schwartz et al., 1965; Van Ravenswaay Claasen et al., 1967).

The synthesis *in vitro* of a specific protein directed by mRNA from mammalian origin has not yet been detected. However, there is enough indirect evidence which supports the idea of the existence of messenger RNA in high organisms.

It is the aim of the present investigation to isolate* and characterize RNA with messenger activity from the calf lens and rat liver. The main characteristic to be used for detecting messenger RNA is the ability of the RNA fraction in question to stimulate the incorporation of amino acids into protein in the cell-free system prepared from *E. coli*.

A review of the present knowledge concerning the mechanism of protein synthesis is given in chapter 2. In addition, attention is focused on reports dealing with homologous and heterologous messenger RNA directed amino acid incorporation into protein in the cell-free system of *E. coli*.

* Part of the results described in this thesis has been published elsewhere. (Konings and Bloemendal, 1969; Bloemendal, Vennegoor and Konings, 1969).

An account of the tissues and methods used for the isolation and characterization of RNA with messenger activity are given in chapters 3 and 4, and in chapter 5 some characteristics of the test system prepared from *E. coli* B are described.

The characteristics of the RNA preparations isolated by different procedures with respect to their stimulatory effect of amino acid incorporation into protein, are described in chapter 6.

In chapter 7 some special characteristics of these RNA preparations such as their base composition and electrophoretic behaviour on polyacrylamide gels, are presented.

In the final chapter, the various results obtained from the present investigations are discussed and related to results described by other investigators.

"It is not impossible to imagine that the primitive machinery had no protein at all and consisted entirely of RNA".

F. H. C. CRICK

CHAPTER II

MECHANISM OF PROTEIN BIOSYNTHESIS

2.1 INTRODUCTION

Extensive reviews on protein biosynthesis have recently been published (Voorma, 1965; Watson, 1965; Campbell et al., 1966; Singer et al., 1966; Bloemers, 1967; Novelli, 1967; AB, 1968; Reinecke, 1968; and Spirin, 1968).

In this chapter a summary of the ideas concerning the mechanism of protein biosynthesis will be given. In addition attention will be focused on reports dealing with amino acid incorporation into protein directed by messenger RNA in the cell-free system of *E. coli*.

2.2. THE MECHANISM OF PROTEIN SYNTHESIS IN *E. COLI*

Our present knowledge concerning the mechanism of protein synthesis is schematically represented in figure 1. The various steps indicated in this scheme will be discussed.

2.3 ACTIVATION OF AMINO ACIDS

Before the amino acids can be incorporated into protein, they have first to be activated by ATP according to reaction a. (Hoagland, 1955; Berg, 1955; Hoagland et al., 1957).

a. Amino acid + ATP + enzyme $\rightleftarrows$ aminoacyl $\sim$ AMP — enzyme + PPi.
b. Aminoacyl $\sim$ AMP — enzyme + tRNA $\rightleftarrows$ aminoacyl-tRNA + enzyme.

Next the activated amino acid is transferred to the terminal adenosine of tRNA according to reaction b. (Hoagland et al., 1958; Zachau et al., 1958). The carboxyl group of the amino acid is linked to the 3'-hydroxyl group of the adenosine (Nathans et al., 1963; Feldman et al., 1964). Both reactions are catalysed by the same enzyme *viz.* aminoacyl synthetase. Each amino

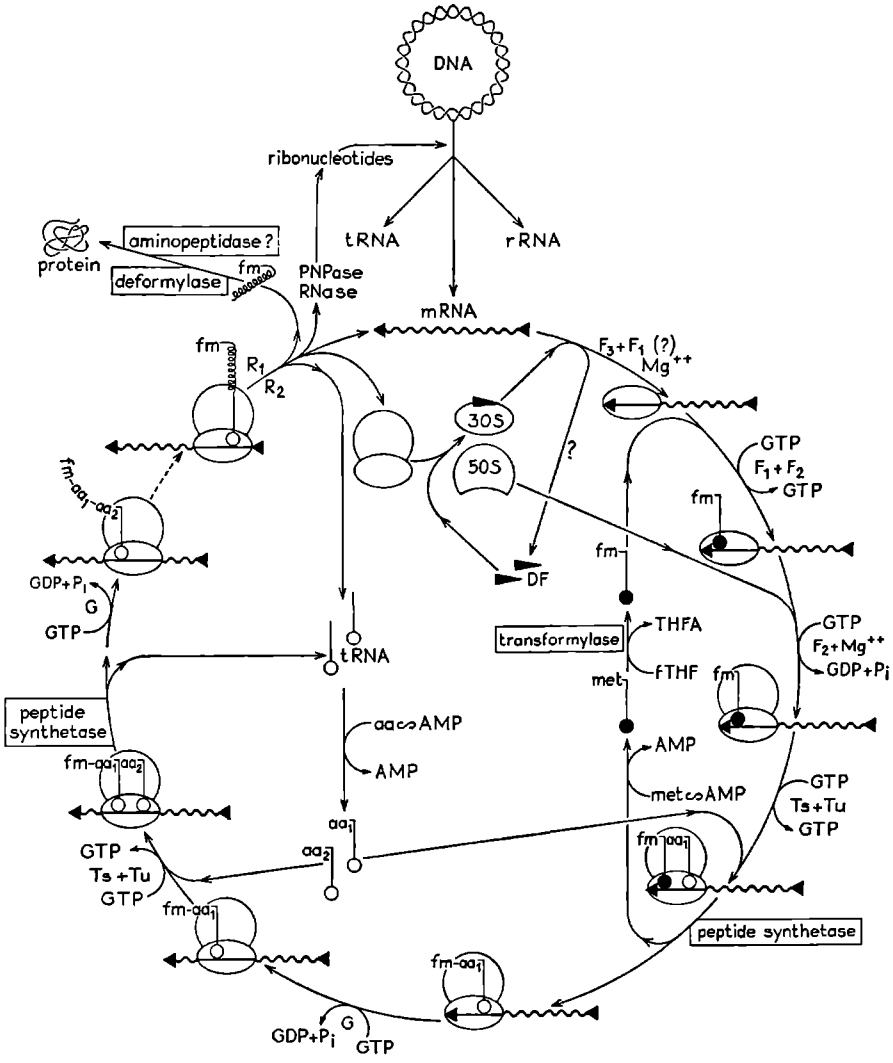

FIGURE 1 Schematic representation of the mechanism of protein synthesis.

acid has its own specific enzyme and corresponding specific tRNA's. (De Moss et al., 1955; Hoagland et al., 1958; Nishimura and Novelli, 1963; 1964a; 1964b).

2.4 INITIATION OF PROTEIN SYNTHESIS

The activated amino acid can now be incorporated into the polypeptide chain. First of all, however, the initiation complex has to be formed.

2.4.1 *Binding of template or messenger RNA to the ribosomes*

The reaction preceding the formation of the first peptide bond involves binding of mRNA to the ribosome (figure 1). Formerly it was thought that initiation was started by binding of the whole ribosome to the mRNA. From more extensive studies it became clear that the first reaction was the binding of the free 30S subunit to the mRNA (Eisenstadt and Brawerman, 1967; Godson et al., 1967). In addition to magnesium ions, a binding factor F_3 or C and probably also a factor F_1, are required for this binding (Eisenstadt and Brawerman, 1966a; Revel and Gros, 1966; Revel et al., 1968; Iwasaki et al., 1968). In *E. coli* the binding of factor F_3 to mRNA probably precedes the binding of the 30S ribosomal subunit (Brown and Doty, 1968). In order to obtain functional proteins the binding between the 30S subunit and mRNA must be a very selective one. The binding site(s) on (polycistronic) mRNA for the ribosomal subunits must have special characteristics. It is still not clear whether the initiator codon alone is enough for this specific recognition process. (See 2.2 and Bretscher, 1968b; Grodzicker and Zipser, 1968).

The close relationship between RNA and protein synthesis in *E. coli* has been known for several years (Stent and Brenner, 1961; Neidthardt, 1964; Stent, 1964; Byrne et al., 1964; Imamoto et al., 1968; Olsnes et al., 1968). Recently it was demonstrated that the synthesis of RNA and of protein in *E. coli* strains with stringent control of RNA synthesis, directly or indirectly depended on the presence of N-formylmethionyl-tRNA (see 2.4.2 and Shih et al., 1966; Ron and Davis, 1966), and that RNA synthesis *in vitro* was stimulated by the addition of ribosomes to the incubation mixture consisting of RNA-polymerase and endogenous DNA (Shin and Moldave, 1966). This stimulation, however, could not be observed in the absence of the so-called translation or binding factor C (F_3). (Revel and Gros, 1966; 1967; Martia et al., 1967). In a later communication Revel et al. (1968) presented evidence that it was the factor C(F_3) dependent

binding of the 30S subunit to the nascent mRNA which was responsible for the stimulation of the transcription process.

These results are possibly an indication that *in vivo* the factors which control the initiation of translation also regulate the transcription process (cf. Edlin and Maaløe, 1966; Mehdi and Yudkin, 1967; Edlin et al., 1968; Forschhammer and Kjeldgaard, 1968; Lavallé and De Hauwer, 1968; Celis and Conway, 1968; Sarkar and Moldave, 1968).

2.4.2 *The binding of N-formylmethionyl-tRNA to the mRNA-30S subunit complex*

Translation of mRNA occurs from the 5'- to the 3' -terminus (Salas et al., 1965; Terzaghi et al., 1965). In addition the synthesis of a protein is a perfectly straightforward process. Therefore it was reasonable to assume that the first codon in an mRNA chain simply specified the first amino acid in the polypeptide chain and that no special starting signal was required. The discovery by Marcker and Sanger (1964) of N-formylmethionyl-tRNA in *E. coli* extracts, however, shed new light on this situation. It was suggested that N-formylmethionine constituted the initial unit of the protein molecule (Noll, 1966).

The Cambridge group was able to separate methionyl-tRNA of *E. coli* into two distinct species, only one of which could be formylated enzymatically (methionyl-$tRNA_F$). (Clark and Marcker, 1966a; Clark et al., 1968; Cory et al., 1968a; 1968b; Dube et al., 1968; Seno et al., 1968; Doctor et al., 1969). Formylation occurs only after the amino acid methionine has become attached to the $tRNA_F$ molecule. (Marcker, 1965; Marcker et al., 1966; Dickerman et al., 1967a; 1967b).

Experiments with natural and synthetic messenger RNA provided evidence that N-formylmethionyl-tRNA functions as the chain initiator and that AUG and GUG, when present at the beginning of the message, are the initiator codons (Adams and Capecchi, 1966; Capecchi, 1966a; Clark et al., 1966a; 1966b; Eisenstadt et al., 1966b; Webster et al., 1966; Clark, 1967; Vinuela et al., 1967; Bretscher, 1968c; Miovic et al., 1968). Moreover, these codons function as "phase selectors" in that they automatically fix the reading frame of the polynucleotide message. However, when present in non-terminal positions of the messenger these triplets code for methionine and valine respectively (Leder et al., 1966a; 1966c; Thach et al., 1966; Sunderarajan et al., 1966; Ghosh et al., 1967a; 1967b; Ohta et al., 1968). Studies on *in vitro* systems have indicated that the methionyl residue of unformylated met-$tRNA_F$, which has a free alpha-amino group, is not incorporated into non-terminal positions of the polypeptide chain (Marcker

et al., 1966). Experiments with the binding factors isolated from *Bacillus stearothermophilus* (Skoultchi et al., 1968) indicate that most aminoacyl-tRNA's including met-$tRNA_M$ do form complexes in the presence of GTP whereas F-met-tRNA and met-$tRNA_F$ do not. These results may account for the above mentioned lack of incorporation of methionyl residues attached to tRNA into non-terminal positions of the polypeptide chain (Ono et al., 1968).

After being formylated, N-formylmethionyl-tRNA is selectively bound to the aminoacyl-site of the mRNA-30S complex. Two ribosomal factors, designated F_1 and F_2, and GTP are required for this binding at low magnesium concentrations (5–8 mM). These initiation factors are probably bound to the 30S subunit and are released when the 70S particles begin translation (Parenti-Rosina et al., 1969).

N-formylmethionyl-tRNA can also be bound to the complex in the presence of the analogue of GTP, GMP-PCP. In this situation, however, after the addition of the 50S subunit to the complex, no reaction with puromycin or with aminoacyl-tRNA is possible. This indicates that N-formylmethionyl-tRNA is linked first to the aminoacyl-site of the ribosome and that GTP hydrolysis is required to translocate the N-formylmethionyl-tRNA from the aminoacyl-site (or puromycin inactive site) to the peptidyl-site (or puromycin active site) of the ribosome. (Stanley et al., 1966; Anderson et al., 1967; Hille et al., 1967; Salas et al., 1967a; 1967b; Ohta et al., 1967; Bretscher, 1968b; Guthrie et al., 1968; Jost et al., 1968; Kolakofsky et al., 1968; Munkundan et al., 1968; Grunberg-Manago et al., 1969). Hydrolysis of GTP is catalysed by the initiation factor F_2, which enzymatic activity is dependent on the binding of N-formylmethionyl-tRNA to the ribosomes.

The dependence on GTP is greatly affected by the magnesium concentration of the incubation mixture. At high magnesium concentration (20 mM) the binding of N-formylmethionyl-tRNA to the mRNA-ribosome complex is independent of GTP (Bretscher and Marcker, 1966; Zamir et al., 1966).

Involvement of N-formylmethionine in the initiation of protein synthesis has also been demonstrated in the bacteria *Bacillus subtilis* (Horikoshi et al., 1967) *Micrococcus lysodeikticus*, *Pseudomonas aerugenosa*, *Bacillus stearothermophilus*, and in the blue green alga *Anacystis nidulans* and in yeast (Marcker and Sanger, 1964; Bachmayer et al., 1968; Ono et al., 1968).

Incorporation of other amino acids blocked in the N-terminal position has also been demonstrated (Haenni and Chapeville, 1966; De Groot et al., 1967; 1968a; Verhoef et al., 1967; Ayuso et al., 1968; Reinecke, 1968;

Laycock and Hunt, 1969). In addition, Lucas-Lenard and Lipmann (1967) demonstrated that at low magnesium concentrations the poly U directed incorporation of N-acetylphenylalanine was strongly dependent on the presence of the initiation factors F_1 and F_2. It was also demonstrated that GTP at low magnesium concentrations was necessary for binding of N-acetylphenylalanine to the poly U-ribosome complex. These results can explain why phenylalanine polymerisation in the presence of poly U and in the absence of an initiator only occurs at high magnesium concentrations. Apparently under these conditions (see also 2.5.2) binding of phenylalanyl-tRNA to the poly U ribosome complex is independent of these initiation factors (Weissbach et al., 1968).

2.4.3 *Binding of aminoacyl-tRNA to the initiation complex*

After joining of the larger subunit (50S) to the F-met-tRNA-30S-mRNA complex and transposition of F-met-tRNA to the peptidyl-site (P-site) of the ribosome it is possible to bind aminoacyl-tRNA to the aminoacyl-site (A-site) of the initiation complex (Nomura et al., 1967a; 1967b). At low magnesium concentrations GTP or GMP-PCP and transfer enzymes are required for this binding (see 2.5.2).

After binding of aminoacyl-tRNA to the initiation complex, the first peptide bond, catalysed by the ribosome-bound peptide synthetase can be formed (see 2.5.2). Binding of aminoacyl-tRNA to ribosomes is inhibited by the antibiotics macrolides and probably also by tetracyclines (Mao et al., 1968; Last et al., 1969).

2.5 ELONGATION OF THE PEPTIDE CHAIN

2.5.1 *Translocation of peptidyl-tRNA from the aminoacyl-site to the peptidyl-site of the ribosome*

After formation of the first peptide bond the peptidyl-tRNA is located at the aminoacyl site (figure 1) of the ribosome-mRNA complex. Formation of a new peptide bond occurs only if peptidyl-tRNA is translocated from the A-site to the P-site of the ribosome. The translocation reaction is catalysed by an enzymatic factor G and requires GTP. At the same time the ribosome moves one triplet along the mRNA. The G-factor is involved in the hydrolysis of GTP. This GTPase activity is ribosome dependent and probably both ribosomal subunits are required for this reaction (Conway and Lipmann, 1964; Nishizuka and Lipmann, 1966a; 1966b; Brot et al., 1968; Erbe and Leder, 1968; Tanaka et al., 1968; Weissbach et al., 1968).

2.5.2 *Binding of aminoacyl-tRNA to the peptidyl-tRNA-ribosome complex*

After translocation of peptidyl-tRNA to the P-site a new aminoacyl-tRNA can be bound to the A-site of the ribosome. This binding is, however, dependent on a soluble factor T and on GTP (Seeds and Conway, 1966; Lucas-Lenard et al., 1967; 1968). The T factor consists of at least two components; a stable component Ts and a heat labile component Tu. A complex between GTP, aminoacyl-tRNA and these factors is formed according to a two step reaction:

$$\text{Tu} + \text{GTP} \overset{\text{Ts}}{\rightleftarrows} \text{Tu} - \text{GTP}$$

$$\text{aminoacyl-tRNA} + \text{Tu} - \text{GTP} \rightleftarrows \text{aminoacyl-tRNA} - \text{Tu} - \text{GTP}$$

The component Ts probably affects the rate of complex formation, whereas Tu determines the amount of the GTP complex (Allende et al., 1967; Gordon, 1967; 1968; Seeds and Conway, 1967; Ertel et al., 1968).

From their experiments with heterologous systems, Ceferri et al. (1968) suggested that Ts may be responsible for the recognition process by which the aminoacyl-tRNA enters its site on the ribosome. During the complex formation GTP hydrolysis does not occur, which may indicate that GTP is involved in the interaction with the ribosome and that the energy obtained after hydrolysis of GTP is used for the translocation reaction. GTP cannot be replaced by GDP, and GMP-PCP has only a slight effect (Lucas-Lenard and Haenni, 1968).

The enzymatic transfer of aminoacyl-tRNA to the ribosomes is stimulated by the ammonium ion and by a sulphydryl compound. The two factors G and T have recently been crystallized and they sediment at 5.3S and 5.11S respectively (Parmeggiani, 1968). Analogous "elongation factors" designated F_{IA}, F_{IB} and F_{II} have been described by Ravel et al. (1967a; 1967b; 1967c; 1968a; 1968b).

A similar pattern of translation factors has been observed in yeast and in mammalian systems. Felicetti and Lipmann (1968) have isolated from rat liver and rabbit reticulocytes two fractions T_1 and T_2 which correspond to the factors T and G from *E. coli*. The second fraction was associated with a ribosome linked GTPase activity. Moldave's group also isolated two enzyme fractions from rat liver (transferase I and II) which were active in catalysing the amino acid incorporation. Binding of transferase II, GTP, monovalent cations, magnesium and a sulphydryl compound were required for the translocation reaction while transferase I and GTP or GMP-PCP in

the presence of ammonium and magnesium-ions were required for binding of aminoacyl-tRNA to the A-site of the ribosomes. Binding of transferase II to ribosomes also occurs in the presence of GMP-PCP, however, for the translocation reaction GTP is specifically required (Fessenden and Moldave, 1963a; Gasior and Moldave, 1965; Sutter et al., 1965; Sutter and Moldave 1966; Ibuki et al., 1966; Rao and Moldave, 1967; Schneider et al., 1968; Ibuki and Moldave, 1968a; 1968b; Skogerson and Moldave, 1967; 1968a; 1968b; 1968c).

Schneir and Moldave (1968) presented evidence that at least three protein fractions from rat liver possess transferase I activity. The biological activity of these proteins appeared to be similar but a difference in their apparent molecular weights and relative specific activity was noted. These results are in contrast to those observed with the transfer factor from *E. coli*. However, similar multiple forms of a transfer factor were isolated from calf liver and yeast (Klink and Richter, 1966; Klink et al., 1967; Richter and Klink, 1967; 1968).

Transfer factors TF_1 and TF_2 in reticulocytes have also been described (Hardesty et al., 1963a; Arlinghaus et al., 1964; Ravel et al., 1966). TF_1 is concerned with the GTP dependent translocation and binding of aminoacyl-tRNA to the ribosome-mRNA complex, whereas TF_2 is involved in the catalysis of the peptide bond formation. The latter enzyme, however, in contrast to the peptide synthetase of *E. coli* and rat liver, is apparently not an integral part of the ribosome (see 2.6.1 and Skogerson and Moldave, 1968a, 1968b, 1968c). TF_1 can be divided into two subfractions, one of which contains ribosome-dependent GTPase activity (Heintz et al., 1966; Arlinghaus et al., 1968; Hardesty et al., 1968; Shaeffer et al., 1968).

In addition to the enzymatic binding of aminoacyl-tRNA, a non-enzymatic binding is also known.

In *E. coli* binding of aminoacyl-tRNA to the P- and A-sites of the mRNA-ribosome complex occurs at high magnesium concentrations in the absence of the initiation and/or transfer factors. This binding is in contrast to the enzymatic binding inhibited by deacylated-tRNA. (Nirenberg et al., 1964; Kaji et al., 1966; Suzuka et al., 1966; Matthaei and Voigt, 1967; Ravel, 1967a; Cannon, 1967; Salas et al., 1967a; Pestka, 1967; 1968a; 1968b; Takeda et al., 1968).

Pestka (1968a) (see also Rychlik, 1967) demonstrated that a dipeptide could also be formed in the absence of these factors and GTP (cf. Suzuka and Kaji, 1967). Polypeptide synthesis, in the presence of the factors Ts, Tu and G, occurs rapidly once the dipeptide has been formed. These results suggest that the rate limiting step in polyphenylalanine synthesis may be

the formation of the initial diphenylalanyl-tRNA, rather than the binding of phenylalanyl-tRNA to the ribosome -mRNA complex. In addition, these and other results explain why polypeptide synthesis can occur on synthetic or natural messenger RNA's deprived of an initiator codon or initiator -tRNA. (Haselkorn and Fried, 1964a; 1964b; Brawerman et al., 1966; Nakomoto et al., 1966).

Non-enzymatic binding of aminoacyl-tRNA to reticulocyte and rat liver ribosomes in the presence of mRNA has also been demonstrated. (Hardesty et al., 1963a; Ibuki and Moldave, 1968b; Shaeffer et al., 1968; Skogerson and Moldave, 1968b). The explanation of these results is sought in the differences in binding at both tRNA sites under anomalous conditions. It is suggested that in the case of enzymatic binding a possible shape change in the region of the binding sites occurs simultaneously with binding of aminoacyl-tRNA. In addition, Shaeffer et al. (1968) found that after incubation of this complex with the transfer enzyme, peptide synthetase (TF-2), no dipeptide could be produced. However, only after extra addition of their binding enzyme (called translocase TF-1) and GTP, could a dipeptide be detected.

These results, which contrast with those of Pestka, (1968a) probably represent a real difference between bacterial and mammalian ribosomes.

After binding of the aminoacyl-tRNA to the peptidyl-tRNA-mRNA-ribosome complex, the formation of a peptide bond occurs. This latter reaction is catalysed by peptide synthetase, which is an integral part of the 50S subunit. The peptide synthetase activity is dependent on monovalent cations and sulphydryl compounds. (Gottesman, 1967; Monro et al., 1967a; Monro, 1967; Maden et al., 1968a; Pestka, 1968a). The possibility cannot be excluded that the conformation of this enzyme, due to the polarity of the chemical reactions catalysed, defines the peptide-site and aminoacyl-site on the 50S particle. Studies with the so-called "fragment-reaction" have revealed that the reaction is optimal at pH 8.5, at a magnesium concentration of 100 mM, and that the reaction is stimulated by the addition of the monovalent cations ammonium and potassium. (Maden et al., 1968a; Maden and Monro, 1968b; Miskin et al., 1968). It was also found that CpCpA, the 3'-terminal end group of tRNA, was necessary for effective interaction of substrate with enzyme. Interaction was also favoured by acylation (at the alpha-aminogroup) of the aminoacyl residue attached to adenosine and was affected by the nature of the amino acid side chain (Monro et al., 1968).

The process of addition of one amino acid to the peptide chain is repeated

again and again until the chain termination signal (see 2.6) is reached. Meanwhile it is possible that several ribosomes have started the reading of the mRNA from the 5'-terminus. Such complexes of one mRNA strand with several ribosomes are called poly(ribo)somes. (Warner et al., 1962; 1963; Mathias et al., 1964).

2.6 POLYPEPTIDE CHAIN TERMINATION

2.6.1 *Release of the polypeptide chain*

The final step in the completion of the polypeptide chain is its release from the tRNA molecule and thus from the ribosome. Biochemical and genetical studies indicated that there are three triplets in the genetic code of *E. coli* which can cause the release of the growing peptide chain. Thus chain termination occurs when the amber triplet UAG, the ochre triplet UAA or the triplet UGA are encountered in the messenger RNA. (Brenner et al., 1965a; 1965b; 1967; Weigert et al., 1965a; 1965b; Sambrook et al., 1967; Last et al., 1967; Zipser, 1967).

There are three possible alternative mechanisms which could achieve chain termination:

a. there are tRNA molecules which specifically recognise the termination codons;
b. there is no tRNA and termination occurs if there is nothing to read these triplets;
c. these triplets are recognised by one or more protein molecules.

Recently Bretscher (1968a) proved that chain termination does not occur if there is an unreadable triplet and hence that the triplets for chain termination must be recognised. It was also suggested that the recognition of the triplets for chain termination is not achieved by an RNA molecule similar to other tRNA molecules. Evidence that a protein factor was involved in chain termination was already presented by Ganoza (1966). These results were confirmed by Capecchi (1967a) who isolated release factor R required for chain termination of the triplet AUG.

More insight into how these triplets are recognised was given by Nirenberg's group. Caskey et al. (1968) introduced a new and simple assay for chain termination. N-formylmethionyl-tRNA is bound to ribosomes in the presence of its appropriate triplet, AUG. The hydrolysis of this bound initiator – tRNA is then assayed. It was found that the rate of hydrolysis of N-formylmethionyl-tRNA could be increased by addition of Capecchi's release factor in the presence of any one of the three trinucleotides which code for chain termination. With the aid of this assay system, they were

able to separate the release factor R into two components (Scolnick et al, 1968) Component R_1 affects hydrolysis of the ribosome bound N-formyl-methionyl-tRNA in the presence of the triplets UAA or UAG, whereas component R_2 will do the same in the presence of the triplets UAA or UGA Previously it was difficult to imagine how one molecule (either tRNA or protein) could recognise the triplets UAG, UAA and UGA, without recognising the triplet UGG, which codes for the amino acid tryptophan These two proteins make the situation much clearer The pattern of codon degeneracy found with R_1 resembles that found with some species of aminoacyl-tRNA, the molecule interacting with A at the third base position of the messenger codons also interacts with G (Nirenberg et al, 1965, Crick, 1966) However, the degeneracy pattern observed with terminator codons for R_2 differs markedly from patterns found with aminoacyl-tRNA In this case an equivalence of A and G at the second base position of codons is found, while only A at the third position is recognised It is now generally believed that the codon used for chain termination *in vivo* is UAA, and that UAG and UGA are rarely if ever used

Cuzin et al (1967) described an enzyme capable of hydrolysing both the ester linkage between N-acetylaminoacyl-tRNA, diphenylalanyl-tRNA, and N-substituted oligopeptidyl-tRNA's It was found that the rate of hydrolysis of N-acetyl derivatives is higher than those of N-formyl derivatives Similar results were found by other investigators (Zamir et al, 1966, De Groot, 1968b, Vogel et al, 1968a) In addition, Paulin et al (1968) observed that the rate of hydrolysis of N-substituted methionyl-$tRNA_M$ is slower than the rate of hydrolysis of the corresponding derivatives of $tRNA_F$ However, this latter result could not be confirmed by other investigators (Vogel et al 1968b) The characteristics of this hydrolytic enzyme (substrate specificity and charge properties) are different from the release factor R (Capecchi, 1967a) which may possibly indicate that this hydrolysing enzyme is not concerned with chain termination Any alternative function of the latter enzyme, however, remains unknown

Termination of protein synthesis can also be achieved with the antibiotic puromycin (Yarmolinsky et al, 1959) This compound forms a covalent bond with the peptide attached to tRNA (Smith et al, 1965) The reaction is under enzymatic control of the peptide synthetase Only aminoacyl-tRNA or peptidyl-tRNA bound to the P-site of the ribosome can react with puromycin, which therefore provides a very useful tool for studying the mechanism of protein synthesis and the effect of antibiotics on the enzymatic action of peptide synthetase (Leder et al, 1966b, Rychlik, 1966, Bretscher

et al., 1966; Bretscher, 1968c; Hershey and Thach, 1967; Monro and Marcker, 1967: Monro and Vasquez, 1967; Schlessinger et al., 1967; Brot et al., 1968; Cannon, 1968).

In contrast to the fact that protein synthesis starts with N-formylmethionine, the overwhelming majority of completed proteins in *E. coli* have methionine, alanine or serine as the amino terminal residue (Waller, 1963; Capecchi, 1966a; Adams and Capecchi, 1966; Pine, 1969). These results suggest that *E. coli* possesses enzymes for the removal of both the formyl group and methionine from the nascent protein chains. A definite answer in one of these cases is given by Fry et al. (1967) and by Adams (1968). These authors showed that in *E. coli* there exists the enzyme peptide deformylase which removes formyl groups from formylmethionyl-peptides and from proteins made *in vitro*. This peptide deformylase is very labile, particularly in the presence of sulphydryl compounds. This property may explain why proteins synthesized *in vitro* contain formylmethionine on the nascent chains. (cf. Livingston and Leder, 1969).

Weissbach et al. (1967) reported the presence of an enzyme in *E. coli* which removes the formyl groups from N-formylmethionine but not from other N-formyl-amino acids. It was suggested that this enzyme might function by cleaving the formyl group from nascent protein or by splitting formylmethionine already released from protein by another enzyme. However, there are strong indications that the enzyme described by Weissbach et al., is the enzyme acetylornithine deacetylase. (Fry et al., 1967; Adams, 1968). The question whether an enzyme is present in *E. coli* which specifically liberates methionine from polypeptide chains still remains unsolved.

2.6.2 *Release of the ribosomes*

Another mechanism which is still not fully understood is the release of ribosomes from the polysomes after finishing the polypeptide chain. Mangiarotti and Schlessinger (1966) concluded from their experiments that the 70S ribosomes observed in extracts of *E. coli* were all products of polysome fragmentation. By lysis of *E. coli* cells under milder conditions only polysomes and ribosomal subunits could be observed. (Schlessinger et al., 1967; Mangiarotti et al., 1967). These results were in agreement with those of Guthrie and Nomura who postulated that ribosome dissociation was an obligatory step in chain initiation. (Nomura et al., 1967a; 1967b; Guthrie and Nomura, 1968; cf. Cundliffe, 1968). These observations

as well as the strong evidence of a rapid exchange between subunits and 70S particles in growing cells (Kaempfer, 1968; Kaempfer et al., 1968) are by no means incompatible with the existence of a pool of free 70S ribosomes. For instance Köhler et al. (1968) have demonstrated that ribosomes are released from the polysomes as free, stable 70S particles and that the dissociation of these units in the cell is subject to a physiological control which maintains an essentially constant pool of subunits. These findings led to the inference that the dissociation step in the ribosome cycle requires stoichiometric complexing with a dissociation factor DF the limited quantity of which in the cell determines the concentration of subunits. Recently Subrumanian et al. (1968) have demonstrated that such a dissociation factor could be extracted from the native 30S-subunit and that this protein could not be found in other ribosomal fractions of *E. coli*. The effect of the DF is antagonised by increasing concentrations of magnesium and probably also by streptomycin. (Luzzatto et al., 1968).

These results may imply that the DF, 70S ribosomes, ribosomal subunits and magnesium are all in equilibrium. If this is the case, then one can propose a model in which the factor causes the dissociation of free 70S ribosomes and is then displaced from the 30S-particle at the formation of the chain initiation complex. At the moment the possibility that the dissociation factor is identical to the initiation factor F_3 described in 2.4.1, cannot be ruled out.

There are several indications that ribosomal subunit exchange also occurs in mammalian systems (Girard et al., 1965; Joklik et al., 1965a; Bishop, 1966; Hogan et al., 1968a, 1968b).
Joklik et al. (1965b) observed that after infection of Hela cells with vaccinia virus, the nascent vaccinia mRNA was combined with the 40S ribosomal particle. The resultant complex was then rapidly incorporated into structures consisting of one messenger bearing a number of 80S ribosomes. Bonanou et al. (1968) demonstrated for the first time a reversible dissociation of ribosomes derived from mammalian tissues. A dissociation of rabbit reticulocyte ribosomes could be achieved in concentrated solutions of KCl, of which the extent of dissociation appeared to be concentration dependent. If suspended in a magnesium free medium, the pellet obtained by differential centrifugation consisted of subunits. On addition of magnesium ions, ribosomes appeared and a considerable proportion of biological activity (20–40%) returned. Addition of the supernatant fraction resulted in further apparent reconstitution of subparticles into ribosomes and polysomes and in a 50–100% restoration of biological activity.

Working with intact cells, Colombo et al. (1968) could demonstrate

changes in ribosome and subunit populations after blocking metabolism by the addition of sodium fluoride alone, or with cycloheximide, an inhibitor of "80S" protein synthesis. A decrease in the concentration of ribosomal subunits was found after treatment with NaF alone, indicating that free subunits are exhausted due to initiation and are not reformed by dissociation of free 80S ribosomes (see also Bishop 1966, 1968). A drop in numbers of polysomes was also demonstrated. NaF apparently blocks dissociation of ribosomes but not initiation of protein synthesis or dissociation of polysomes. However, treatment with cycloheximide before treatment with NaF prevents the dissociation of polysomes and initiation of new peptide chains, and consequently the concentration of subunits remains more or less constant. If NaF is removed, subunits reappear and precede the restoration of normal protein synthesis. Energy seems to be required for the dissociation of the 80S ribosomes. These results confirm the idea that in eukaryotic cells there is an exchange of ribosomal subunits in the course of protein synthesis.

2.6.3 *Degradation of mRNA*

After having functioned as template for protein synthesis *in vivo*, bacterial mRNA is degraded rapidly.

Recently it was found that degradation begins at the 3'-end of the mRNA molecule and progresses to the 5'-end. (Baker et al., 1968). Exonucleases which act in this direction are RNase II and PNPase. (Thang et al., 1967; Nossal et al., 1968; Klee et al., 1968). The latter enzymes are probably attached to the 30S ribosomes (Natori et al., 1967). Activity of these enzymes could be detected in cell-free extracts of *E. coli* (Wade and Lovett, 1961; Futai et al., 1966; Artman et al., 1967). It was, however, not certain whether these enzymes also acted *in vivo* in the same way, as they may belong to different cell structures. Experiments with RNase I (an endonuclease) deficient mutants and with temperature sensitive mutants for RNase II, indicated that RNase II is involved in the inactivation of mRNA. However, they provide no evidence that PNPase may also be involved, but do not eliminate such a possibility. (Sarkar and Dürwald, 1966; Mizuno and Anraku, 1967; Kivity-Vogel et al., 1967; 1968; Castles and Singer, 1969).

2.7 INITIATION OF PROTEIN BIOSYNTHESIS IN HIGHER ORGANISMS

The mechanism of chain initiation in higher organisms is still not clear.

Attempts to demonstrate that initiation on 80S ribosomes involves N-formylmethionine have been unsuccessful (Rich et al., 1966; Summers

and Maizel, 1967). Caskey et al. (1967) could demonstrate that one of two methionyl-tRNA species of guinea pig liver could be formylated with a transformylase preparation from *E. coli* (cf. Takeishi and Ukita, 1968). Attempts to formylate methionyl-tRNA with a variety of mammalian extracts were, however, unsuccessful. In contrast, Smith and Marcker (1968) have recently demonstrated that protein synthesis in mitochondria from rat liver and yeast is initiated by N-formylmethionyl-tRNA (cf. Galper and Darnell, 1969). These results and the knowledge that protein synthesis in chloroplasts of *Euglena gracilis* is also initiated by N-formylmethionine (Schwartz et al., 1967), may imply that initiation of protein synthesis by N-formylmethionyl-tRNA is a characteristic of the 70S type ribosomes (see also Parisi et al., 1967).

Chain initiation on 80S ribosomes has frequently been studied in the reticulocyte system because about 80% of the protein synthesized in the reticulocyte is haemoglobin, and in addition both alpha and beta chains have valine as N-terminal residue. Several investigators have given indirect evidence that haemoglobin is synthesized sequentially beginning with the N-terminal valine residue. (Bishop et al., 1960; Dintzis, 1961; Hardesty et al., 1963b; Arnstein et al., 1964; Bishop, 1964; 1966; 1968; Lamfrom and Knopf, 1964; Rahaminoff and Arnstein, 1967). However, more work has to be done in order to obtain definite proof that no other specific chain initiation mechanism is involved.

2.8 PROTEIN SYNTHESIS IN THE CELL-FREE SYSTEM OF *E. COLI*

Considerable success has been achieved with cell-free *E. coli* extracts (Nirenberg and Matthaei, 1961) in unravelling the genetic code. The remarkable response of this system to a variety of synthetic poly-ribonucleotides with random or ordered sequences of nucleotides (Nirenberg et al., 1961; 1963; Speyer et al., 1963; Khorana, 1965; Nishimura et al., 1965; Grünberger et al., 1966), has made the *E. coli* system the standard test for messenger activity of RNA preparations isolated from all kinds of organisms.

There are, however, only a few examples in the *E. coli* system of the promotion of the synthesis of a specific protein by the addition of a specific natural mRNA.

Nathans et al. (1962) could demonstrate for the first time that RNA isolated from the bacteriophage f2 stimulated the synthesis of the phage coat protein. Similar results have been obtained with RNA isolated from the closely related phages MS2 (Nathans, 1965) and R17 (Yamazaki et al., 1966) and Qβ (Bassel, 1968). Also messenger RNA from T_4-infected cells

stimulated the synthesis of phage-specific lysozyme (Salser et al., 1967). In addition the phage -RNA *E. coli* system embodies all the factors required for the initiation, termination and release of polypeptide chains; it responds to the "punctuation marks" of the messengers and even suppression could be observed *in vitro*, indicating a high degree of fidelity (Engelhardt et a., 1965; Capecchi, 1966b; Capecchi, 1967a; 1967b; Eggen et al., 1967; Bretscher, 1968d; Lodish, 1968).

Nirenberg and Matthaei (1961) were the first investigators to test the messenger activity of heterologous (TMV) RNA in the cell-free system. At the beginning it was thought that the viral RNA was stimulating the synthesis of viral coat protein (Tsugita et al., 1962). However, more searching experiments proved that this was not the case (Aach et al., 1964; Schwartz, 1967). Comparable results were obtained with RNA preparations isolated from the BMV (Stubbs and Kaesberg, 1967). Although very high incorporations are obtained with TYMV-RNA, the synthesis of a specific TYMV protein has not yet been detected (Voorma, 1965; van Ravenswaay Claasen, 1967).

Specific protein synthesis in the *E. coli* system directed by heterologous RNA has only been obtained in three cases:

a. RNA isolated from STNV promoted the synthesis of a protein with an identical tryptic finger-print pattern to the phage coat protein itself (Clark et al., 1965).

b. Similar results could be obtained after addition of AMV-RNA to the cell-free system (van Ravenswaay Claasen et al., 1967).

c. Very recently it was found that addition of an RNA preparation from rabbit reticulocytes, produced a material having properties of rabbit globin. The presence of N-acetylvalyl-tRNA seemed to be essential (Laycock and Hunt, 1969).

The RNA's from the STNV and AMV are presumably monocistronic, indicating that only their own coat protein can be synthesized. Failure of both BMV and TMV-RNA to direct specific protein synthesis can possibly be explained by assuming a difference in initiation mechanism on the ribosomes of bacteria (70S) and plant cells (80S). Specific coat protein synthesis could, however, be detected for STNV and AMV. These results probably indicate that translation of STNV-RNA and AMV-RNA in plant cells occurs on "bacterial-like" ribosomes. Ribosomes in plant cells which may have this function are the ribosomes of the chloroplasts (Eisenstadt and Brawerman, 1964; Schwartz et al., 1965; Stutz and Noll, 1967), for it has been demonstrated that the chloroplasts have the same initiation

mechanism as has *E. coli* (Schwartz et al., 1967). Moreover, specific coat protein synthesis of bacteriophage f2 in cell-free extracts of *Euglena gracilis* has also been described (Schwartz et al., 1965).

A number of laboratories have recently reported progress in the isolation of RNA with messenger activity from mammalian tissues. The results of these investigations are summarised in tables 1, 2 and 3. In addition the relative incorporation of different amino acids directed by mammalian RNA are given in table 4.

From these data it can be concluded that there is no obvious correspondence between the results obtained by several investigators. Some results are even contradictory and the synthesis of a specific protein directed by mammalian RNA has only been detected in one case. The meaning of these observations will be discussed in chapter 8 in connection with the results described in this dissertation.

TABLE 1. Survey of investigations on messenger activity in RNA preparations isolated from rat liver

Method of RNA isolation	Subcellular fraction	^{14}C-Amino acid tested	Magnesium conc. (mM)	G-C %	Relative stimulation	Sedimentation value with highest messenger activity	References
Phenol, 0.1% SDS pH 7.0, 20°C.	nuclei	val	10	—	—	19S and >40S	Barondes et al. (1962a)
	microsomes	val	10	—	—	—	„
Phenol, pH 8.5, 25°C.	nuclei	leu	10	55.6	13 – fold	—	Brawerman et al. (1963)
Phenol, pH 9.0, 25°C.	ribosomes	leu	10	64.0	3 – fold	—	„
Phenol, 0.1% SDS pH 7.0, 20°C.	nuclei from control and	phe	10.5	—	5 – fold	—	Loeb and Gelboin (1963)
	methylcholantrene treated rats	phe	10.5	—	6 – fold	—	„
Phenol, 1% SDS, 0.5% NDS, pH 7.6, 5°C.	nuclei	val	12	—	10 – fold	20S	Di Girolamo et al. (1964)
	cytoplasm	val	12	—	2.7 – fold	18S	„
	microsomes	val	12	—	1.5 – fold	18S	„
2M LiCl	polysomes	protein hydrolysate	10	60.9	2 – fold	—	Otsuka (1964)
Phenol, pH 9.0, 4°C.	600 g supernatant	leu	10	—	—	10S and 20S	Brawerman et al. (1965)
and at 30°C.	600 g supernatant	leu	10	—	—	16S and 28S	„

Phenol, pH 6.0, 45°C.	nuclei	leu	10	—	48 – fold	—	artouzou et a. (1965)
Phenol, pH 6.0 55°C.	nuclei	leu	10	—	78 – fold	—	„
Phenol, 0.5% SDS pH 7.6, 20°C.	nuclei	val	12	—	9 – fold	—	Liao (1965)
	microsomes	val	12	—	2.7 – fold	—	„
Phenol, 1% SDS, 0.5% NDS, pH 7.6, 5°C.	nuclei from protein de-	val	12	—	—	>28S	Di Girolamo (1966)
	pleted and control rats	val	12	—	—	8S	„
Phenol, 1% SDS, pH 7.0, 20°C.	nuclei from control and	val	10	—	RNA fractions have the same	—	Garren et al. (1966)
	hydrocortisone treated rats	val	10	—	specific activity	—	„
Phenol, 0.25% SDS, pH 7.0, at 45°C and at 65°C.	'phenolic -nuclei'	leu	14	—	2 – fold	—	Hadjiolov (1966)
		leu	14	—	6.5 – fold	—	„
Phenol, 39°C, pH 8.3	'phenolic -nuclei'	leu	10	<60	5 – fold	21S	Hadjivassiliou and Brawerman (1966)
Phenol, pH 7.6, 39°C.	cytoplasm	leu	10	—	—	>28S	„
Phenol, pH 8.3, 39°C.	nuclei	leu	12	55.3	—	—	Hadjivassiliou (1966)
Phenol, pH 7.6, 39°C.	nuclei	leu	12	60.8	—	—	„
Phenol, pH 8.3, 39°C.	'phenolic -nuclei'	phe	10	<60	3.5 – fold	—	Silversteyn and Bondy (1966)

TABLE 1 (continued) Survey of investigations on messenger activity in RNA preparations isolated from rat liver

Method of RNA isolation	Subcellular fraction	^{14}C-Amino acid tested	Magnesium conc (mM)	G-C %	Relative stimulation	Sedimentation value with highest messenger activity	References
Phenol, 1% SDS, pH 7.2, 2°C	polysomes	val	12	—	—	18S	Di Girolamo et al. (1967)
	monosomes	val	12	—	—	18S	„
Phenol, pH 7.6, 0°C.	nuclei	leu	12	60.8	1.5 – fold	4S	Hadjivassiliou and Brawerman (1967)
Phenol, pH 8.3, 38°C.	nuclei	leu	12	55.3	6 – fold	16S and 24S	„
Phenol, pH 7.6, 0°C	cytoplasm	leu	12	—	2 – fold	18S and 28S	„
Phenol, pH 8.3, 38°C	cytoplasm	leu	12	48.1	6 – fold	16S, 28S and >30S	„
Phenol, 1% SDS, pH 5.2, 65°C.	nuclei	phe	12	59.7	2.2 – fold	>30S	Bondy and Roberts (1968)

TABLE 2 Survey of investigations on messenger activity in RNA preparations isolated from rabbit reticulocytes

Method of RNA isolation	Subcellular fraction	Amino acid tested	Magnesium conc (mM)	G-C %	Relative stimulation	Sedimentation value with highest messenger activity	References
Phenol, 1% SDS, pH 7 8, 4°C	polysomes	val	15	—	4 – fold	equal activity in all fractions	Hardesty et al (1963a, 1963b)
Phenol, 0 2% SDS, pH 7 8	polysomes	protein hydrolysate	10	56 4 (17S)	4 – fold	17S	Drach and Lingrel (1964, 1966a)
Phenol, 1% SDS, pH 7 8, 4°C	polysomes	val	17–20	—	7 – fold	—	Shaeffer et al (1964)
Phenol, 1 2% SDS, pH 7 3, 4°C	whole cells	protein hydrolysate	10	—	1 – fold	—	Willson and Gros (1964)
Phenol, pH 9 0, 4°C	supernatant 10,000 g	leu	10	—	3 – fold	15S and 30S	Brawerman et al (1965)
Phenol, 0 2% SDS, pH 7 8	polysomes	leu	13	—	2 6 – fold	—	Drach and Lingrel (1966b)
Phenol, m Cresol, 0 5% SDS, pH 7 6, 20°C	supernatant 30,000 g	13 ^{14}C-amino acids	5	55 2 (8S)	—	8S (synthesis of material resembling rabbit globin was demonstrated)	Laycock and Hunt (1969)

TABLE 3. Survey of investigations on messenger activity in RNA preparations isolated from different animal tissues.

Source of RNA	Method of RNA isolation	Subcellular fraction	Amino acid tested	Magnesium conc. (mM)	G-C %	Relative stimulation	Sedimentation value with highest messenger activity	References
Hela cells	Phenol, 0.5% SDS, pH 5.1, 60°C	whole cells	val	10	59.5	4.5 – fold	45S	Scherrer et al. (1963)
Sheep thyroid	Phenol, pH 6.0, 45°C	nuclei	leu	10	—	66 – fold	>28S	Cartouzou et al. (1965)
	Phenol, pH 6.0, 55°C	nuclei	leu	10	—	70 – fold	—	„
Rat prostate	Phenol, 0.5% SDS, pH 7.2,	nuclei	val	12	62	11 – fold	—	Liao (1965)
		polysomes	val	12	62	3 – fold	—	
Spleen and lymph nodes	Phenol, 0.5% SDS, pH 5.0, 65°C	whole cells	val	12	—	5 – fold	9S and >34S	Mach and Vassalli (1965a)
Spleen and lymph nodes	Phenol, 0.5% SDS, pH 5.0,	whole cells						
	at 20°C		val	12	—	1 – fold	equal activity	Mach and
	45°C		ser	12	—	12 – fold	in all fractions	Vassalli (1965b)
	65°C		ser	12	—	31 – fold		
RPC-20 plasma cell tumor	LATC, $MgSO_4$, SDS, pH 7.4, 3°C	microsomes	mixture of 16 amino acids	15.5	—	2.4 – fold	18S	Kuff and Hymer (1966)
Human placenta	Phenol, pH 8.3, 39°C	nuclei	phe	10	<60	2 – fold	—	Silversteyn and

Fl-cells	Phenol, 0.5% SDS, pH 8.1, 0°C MAK-column chromatography	whole cells	protein hydrolysate	17.5	—	stimulation linear with amount of RNA added	—	Yoshikawa-Fukada (1966)
Walker tumor cells	not indicated	nuclei	arg	7	—	13.3 – fold	18S	Jacob and Busch (1967)
	not indicated	nuclei	arg	7	—	1.9	equal activity in all fractions	„
KB-cells	Phenol, 0.5% SDS, pH 7.2, 20°C	polysomes	leu	10	—	2.1 – fold	<28S	Ristow and Köhler (1967)
		monosomes	leu	10	—	1.6 – fold	<28S	
		subribosomal particles	leu	10	—	1.5 – fold		
Sheep thyroid	Phenol, pH 6.0	crude	leu	14				Cartouzou et al. (1968)
	at 4°C	nuclei			56	2 – fold	—	
	45°C				54.4	17 – fold	—	
	55°C				51.7	18 – fold	—	
	65°C				45.9	16 – fold	—	
	85°C				39.9	14 – fold	—	
Sheep thyroid	Phenol, 1% SDS, pH 6.0, 0°C	polysomes	leu	14	—	—	10S and >30S	Cartouzou et al. (1968)
Rat brain	Penol, 1% SDS, pH 5.2, 65°C	nuclei	phe	12	58.6	8 – fold	26S	Bondy and Roberts (1968)
Rat brain	Phenol, 1% SDS, pH 7.6, 5°C	ribosomes	phe	12	61.2	1.3 – fold	—	„

TABLE 4. Ratios of incorporation of different amino acids in the presence and absence of RNA

RNA fraction		ile	val		References
Reticuloc. RNA		1	2		Hardesty et al. (1963a)
		ile	val		
Endogenous		1	1.54		Shaeffer et al.
Reticuloc. RNA		1	2.05		(1964)
	leu	ile	val		
Endogenous	1	0.096	0.49		Brawerman et al.
Reticuloc. RNA	1	0.16	0.21		(1965)
	val	phe	leu	lys	
Endogenous	1	2.68	3.17	1.01	Liao (1965)
Prostate RNA	1	1.02	0.69	0.69	
	ser	thr	pro		
Endogenous	1	1.36	1.07		Mach and Vassalli
Spleen RNA	1	0.65	0.73		(1965b)

Values indicate relative incorporations.

AN ACCOUNT OF THE TISSUES AND METHODS USED FOR THE ISOLATION AND CHARACTERIZATION OF RNA WITH MESSENGER ACTIVITY

3.1 INTRODUCTION

Tissues which are the most useful for studying messenger RNA are those which manufacture specific proteins in a high quantity. These tissues can roughly be divided into two groups:

a. tissues which have a high rate of protein synthesis as well as a high rate of RNA synthesis (for example liver).

b. tissues which have in addition to a relatively high rate of protein synthesis, a low or zero rate of RNA synthesis (for example lens or reticulocytes).

For the isolation and characterization of RNA with messenger activity we have studied one tissue from each group, namely the calf lens and rat liver. First, some characteristics of these tissues will be described.

3.1.1 *Description of some main characteristics of the lens*

The lens is an entirely epithelial structure. It is completely enclosed by the capsule, an elastic non-cellular membrane (10–20 micron thick), which is believed to be secreted by the epithelium (Bakker, 1937). The membrane is composed of protein and polysaccharide (Pirie, 1951), and is possibly concerned with the transport of ions.

The lens develops from the thickening of the surface ectoderm, which first forms the lens placode (figure 2). Next the lens vesicle is formed, which then separates from the surface ectoderm to lie in the mouth of the optic cup. Finally there is an obliteration of the internal cavity by growth of the lens cells from the posterior wall. When these cells have filled the lenticular space they stop growing (Reeder and Bell, 1967).

The further growth which is the result of division in the single layer of epithelium at the anterior surface is described in figure 3 (Papaconstantinou, 1967).

The epithelial cells present on the anterior surface of the lens can be divided into two distinct regions: the germinative region and the central region (see figure 3). The germinative region is composed of cells which

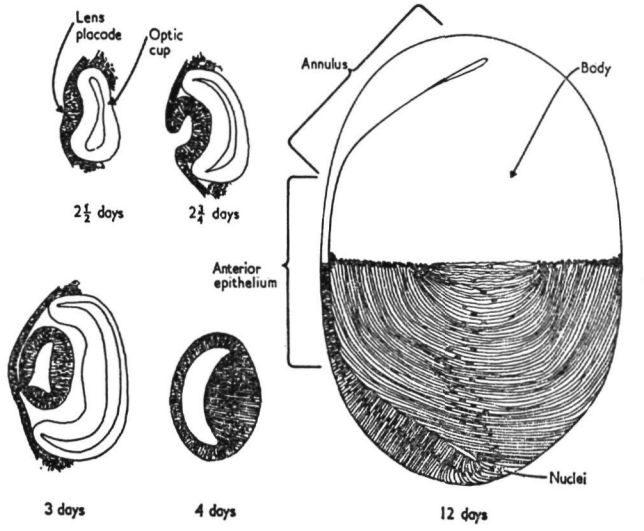

FIGURE 2. Schematic representation of the development of the lens. (Reproduced from R. Reeder and E. Bell, 1967).

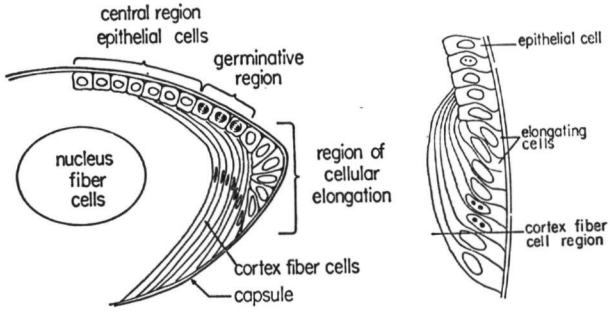

FIGURE 3. Diagrammatic representation of the lens of the adult vertebrate. (Reproduced from J. Papaconstantinou, 1967, with permission).

have a high mitotic rate relative to the cells in the central region. As the animal ages, mitosis becomes negligible in the central zone and is essentially limited to the germinative region, although even in this region it is significantly reduced (figure 3). On the equatorial side of the germinative epithelial cells is the region of cellular elongation. Transition from the germinative region to the equatorial region is accompanied by cell differentia-

tion. This cell differentiation consists of a transition from the more or less cuboidal epithelial cells to the elongated fiber cell. Fiber cells are continuously formed throughout the prenatal and postnatal life of the animal. Consequently the lens is composed of layer upon layer of the fiber cells and the continuous formation of these accounts for the growth of this tissue.

The cytological and cytochemical alterations which can be observed during this cell differentiation from cuboidal cell into fiber cell are summarized in table 5.

TABLE 5. Cytological and cytochemical alterations which can be observed during cell differentiation from cuboidal cell into fiber cell

Cuboidal cell	Fiber cell
Cells in active growth phase	Cells in stationary growth phase
Nuclei and nucleoli normal	Nuclei and nucleoli decrease in size and ultimately vanishe
Rough endoplasmatic reticulum	Smooth endoplasmatic reticulum
Basophilic staining properties	Acidophilic staining properties (Cogan, 1962)
Ribosomal population is high	Ribosomal population is low (Eguchi, 1964; Karasaki, 1964)
DNA and RNA synthesis	No DNA and RNA synthesis (Reeder and Bell, 1965)
α, β-crystallin synthesis is inhibited by actinomycin D	α, β-crystallin synthesis is stimulated by actinomycin D (Papaconstantinou 1964, 1967; Papaconstantinou et al., 1964, 1966; Stewart and Papaconstantinou, 1967a, 1967b)
Messenger RNA (for crystallins) is unstable	Messenger RNA (for crystallins) is stable (Scott and Bell, 1964, 1965; Reeder and Bell, 1965, 1967; Stewart and Papaconstantinou, 1967a, 1967b)
No γ-crystallin synthesis	γ-crystallin synthesis (Papaconstantinou, 1965, 1967)

The main reasons for beginning an investigation on messenger activity in RNA preparations isolated from calf lens were that:

a. the lens contains highly stable messenger RNA (see table 5);

b. a very high percentage (86.5%) of the proteins in the lens are the specific proteins, crystallins (Krause, 1934);

c. we already have in our laboratory methods for preparing polysomes and have obtained satisfactory results using a protein synthesizing cell-free

system from the calf lens (Bloemendal et al., 1966b; 1968; Schoenmakers et al., 1967; Benedetti et al., 1968; see also Spector and Travis, 1966).

3.1.2 *Some characteristics of the liver*

The liver can roughly be defined as a continuous mass of parenchymal cells (90% of the liver weight), tunnelled by vessels through which blood and excretory products flow. It is developed from two distinct primordia, the hepatic diverticulum of endoblastic origin and the vascular network which develops precociously between the vitelline veins, and to which are added vascular elements of umbilical vein origin. One of the main functions of the liver is to produce plasma proteins. Serum albumin especially is synthesized in a high quantity (in man about 12 g daily; in rat about 200 mg daily). In the adult liver there is a high rate of RNA synthesis while there is a low rate of DNA synthesis (Rouiller, 1964).

The main reasons for using rat liver were;

a. the fact that the liver is easily and quickly removed from the animal;

b. a specific protein (albumin) is synthesized at a high rate and in high quantities;

c. as the possibility for isolation of RNA with messenger activity from rat liver had already been described in the literature (cf. table 1), we started to look for a resemblance in messenger activity and other characteristics between RNA preparations isolated from calf lens and rat liver cells.

3.2 ISOLATION AND CHARACTERIZATION OF RIBONUCLEIC ACID FOR STUDIES IN PROTEIN BIOSYNTHESIS

3.2.1 *Introduction*

It has already been mentioned in chapter 2 that there are three main classes of RNA, namely ribosomal RNA, transfer RNA and template or messenger RNA. However, it is not always easy to assign a particular RNA to one of these classes. One example is an RNA having a base composition of rRNA but with a higher molecular weight (Perry, 1962; Hiatt, 1962; Scherrer and Darnell, 1962). This type of RNA is now characterized as a precursor of rRNA (Muramatsu et al., 1966a; Perry, 1967). On the other hand an RNA may have some characteristics of mRNA but is not a template in protein biosynthesis (Spiegelman, 1961), or an RNA which resembles tRNA in base composition but does not form aminoacyl-tRNA (Comb and Katz, 1964; Zehavi-Willner et al., 1966; Marcot-Queiroz et al., 1965a; Watson and Ralph, 1966; Galibert et al., 1967).

To regard an RNA as ribosomal one should in practice demonstrate its

typical base composition (G + C / A + U = 1.6 in higher animals) and its typical two component sedimentation pattern with peak values of 16–18S and 28–30S for the slower and faster components respectively (Henshaw 1964; Perry, 1967). In addition rRNA is, in contrast to mRNA, methylated (Moore, 1966c; Muramatsu et al., 1968).

To classify an RNA as tRNA one should demonstrate its low molecular weight (sedimentation value 4S) and its typical base composition rich in G-C nucleotides. A rapid turnover of CCA end groups and the presence of unusual minor nucleotides, are also characteristics of tRNA (Rosset and Monier, 1965; Kin-Ichiro, 1967). However, the main functional characteristics of tRNA are its ability to form aminoacyl derivatives and to transfer amino acids to ribosomes (Hoagland, 1958).

The characterization of messenger RNA is much more complicated. The main criterion for messenger RNA is its ability to direct the synthesis of a particular well-defined protein in a heterologous cell-free system. Until now such unequivocal proof has been extremely laborious and usually impossible (see 2.8). Therefore a search must be made for some less direct evidence.

The main criteria which are normally used in the literature for proving the presence of messenger RNA are:

a. the RNA under investigation must have a base composition which is different from ribosomal RNA and which resembles the base composition of homologous DNA (G + C / A + T $\approx$ 0.7–0.8);

b. the RNA should have the ability to hybridise with this DNA;

c. the RNA should stimulate amino acid incorporation into protein in a cell-free system containing preincubated ribosomes;

d. from the theoretical point of view it can be expected that most of the messenger RNA has a molecular weight and sedimentation value which is different from rRNA and/or tRNA.

The criteria which we have used to characterize our RNA preparations with respect to messenger activity are those described above under a, c and d respectively.

A remark about the first criterion should be made. Since Jacob and Monod (1961) postulated that the existence of messenger RNA was necessary for the continuous flow of information from the nuclear DNA to other places in the cell, many investigators have tried to isolate RNA with a DNA-like base composition. Most of these attempts were, however, unsuccessful.

The failure to find such an RNA could be explained by the following considerations:

a. only one of the DNA strands is transcribed into RNA (Spiegelman and Doi, 1963). If this is correct, then only if both DNA-strands have the same base composition could one expect to find RNA with DNA-like base composition. However, experimental evidence which would justify this assumption is lacking.
b. only part of the nuclear DNA is transcribed into RNA at one time or, at least, in a very short period of time.
For these reasons one is very unlikely to find RNA with DNA-like base composition. One is more likely to find an RNA fraction with a base composition which is different from rRNA and/or from tRNA.

3.2.2 *Isolation of RNA with messenger activity*

With the exceptions of messenger RNA present in RNA phages and viruses, it is extremely difficult to isolate messenger RNA completely free from other nucleic acids. In most cases methods which are suitable for extraction of stable RNA from cells will also yield messenger RNA but a number of variations have to be made which made the ultimate fractionation easier. A brief review of the methods used for extraction of RNA will now be given.

High salt concentrations have been used for the extraction of RNA from different tissues (Allfrey and Mirsky, 1957; Hotta and Osawa, 1958; Kit, 1960). All methods based on this principle have several drawbacks. A lengthy procedure for the extraction of RNA without adding RNase inhibitors involves the risk of enzymatic damage of RNA.

In 1956 Kirby introduced the "phenol extraction" procedure for the isolation of RNA. Phenol is very effective in denaturing proteins. It inhibits the activity of cellular RNases and at the same time leaves the native structure of the nucleic acids intact. Later many investigators improved the original phenol method of Kirby by several additions to the water and/or phenol layer (Kirby, 1965; Wilkinson and Kirby, 1966; Girard et al., 1965; Fujisawa and Muramatsu, 1968).
With the aid of labelling techniques it was found by Sibatani et al., (1959; 1962) and by Georgiev et al., (1960; 1962; 1963) that not all cellular RNA was extracted by the original phenol method but that a portion of the RNA remained bound at the water phenol interphase. The material at the interphase consisted of cell nuclei (so-called "phenolic nuclei") and the RNA non-extractable by phenol treatment was nuclear RNA, or more precisely RNA of chromosomes together with RNA of nucleoli.

Sibatani et al. (1962) observed that after treatment of these nuclei with

1 M NaCl, which destroyed the nuclear structures, an RNA fraction could be isolated which was highly labelled and which had a base composition with a G-C content much lower than that of ribosomal RNA.

Georgiev and Mantieva introduced the "hot phenol" fractionation technique for the isolation of nuclear RNA. When nuclei were treated sequentially with a phenol -0.14 M NaCl mixture (pH 6) at different temperatures the following results were obtained: at temperatures lower than 40–50°C rRNA was mainly released, while at 50–55°C a mixture of rRNA and DNA-like RNA was extracted. Treatment of the phenolic nuclei with a hot (60–65°C) phenol -NaCl (0.14M) mixture, resulted in about 80–90% extraction of the nucleochromosomal RNA which had a base composition between that of rRNA and DNA. The interphase, after the final extraction, contained a small amount of RNA (about 10% of the amount in phenolic nuclei). This RNA could be extracted by treatment at 85°C in the presence of SDS and appeared to be identical to the RNA extracted at 60–65°C. Similar methods have been applied to different tissues by several other investigators (Bruns et al., 1965; Bukrinskaya et al., 1966; Mahler et al., 1966; Coolsma and Gruber, 1968).

Instead of the extraction of RNA at different temperatures, Hadjivassiliou and Brawerman (1965) introduced an extraction procedure which depended on extraction of RNA at different pH values. These authors found that RNA extracted with slightly alkaline buffers ("high pH RNA"; pH 8–9) had the highest messenger activity and had a base composition which was different from ribosomal RNA.

We decided to use a method for the extraction of RNA from whole tissues, and from crude nuclei, which was based on a combination of the "hot phenol" and the "high pH" extraction procedures.

In short, the tissues were first extracted several times at pH 7.6 and 30–40°C, then an extraction at pH 8.3 and 65°C was performed. To avoid aggregation of RNA the extraction was performed in the presence of EDTA. The details will be described in the experimental section.

Several methods have also been described for the isolation of RNA with messenger activity from subcellular cytoplasmic particles (compare tables 1, 2 and 3). Most methods depend on extraction of RNA with phenol in the presence of detergents (e.g. SDS) and additional RNase inhibitors such as NDS and PVS (Kirby, 1962; Möller and Boedtker 1962). In addition, a chemical (8-hydroxychinoline) which minimises oxidation of phenol, and which may be valuable in removing metal ions involved in binding RNA to protein, is sometimes added to the extraction mixture (Kirby, 1962).

The extractions are frequently performed at low temperatures. The method we have used was a combination of methods which had proved to give fairly high yields of RNA with messenger activity.

Details of the method used will be given in the next chapter.

3.2.3 *Characterization and fractionation of RNA with messenger activity*

The best measure of messenger activity of RNA preparations available at present, is the ability of the RNA preparations in question to stimulate the amino acid incorporation into protein in a heterologous cell-free system. To characterize our RNA preparations on messenger activity we used the cell-free system of *E. coli* B, prepared according to a slightly modified method of Nirenberg and Matthaei (1961).

Isolated RNA fractions with messenger activity are usually heavily contaminated with rRNA and/or tRNA. A further fractionation of RNA may be useful but this is limited by the fact that the major part of RNA is built up of four different nucleotides and that the charge per nucleotide unit is nearly the same at neutral pH. In addition, the phospho-diester bond in RNA is very sensitive to extreme pH values. It can also be expected that messenger RNA (in contrast to rRNA and tRNA) in these RNA preparations, has a heterogeneous molecular weight. Using other characteristics (e.g. sedimentation, electrophoretic behaviour) many suitable methods have been developed for fractionation of RNA preparations with messenger activity at neutral or nearly neutral pH's (Wake and Baldwin, 1962; Kidson and Kirby, 1965; Tsanev et al., 1966a; Lichtenstein et al., 1967; Young, 1968; Ellem and Rhode, 1969). The method which, in our hands, appeared to be most useful for fractionation of high molecular weight RNA, was the procedure described by Britten and Roberts (1960). This method depends on the sedimentation behaviour of RNA in sucrose density gradients. By centrifugation the RNA molecules, which have a different molecular weight, are separated on the basis of their sedimentation value.

Recently methods were described for separation of high molecular weight RNA by means of electrophoresis on polyacrylamide gels (Richards and Gratzer, 1964; Loening, 1967; Weinberg, 1967; Dingman et al., 1968; Bishop et al., 1967). The latter method depends on the molecular sieve properties of the gel. Unfortunately this method is only feasable for analytical purposes. Extraction of RNA from these gels on a preparative scale with at least a reasonable recovery is not possible at present (see 4.6).

Separation of high molecular weight RNA from low molecular weight RNA (4 and 5S) and DNA can be achieved by making use of their differences

in solubility in high salt concentrations. High molecular weight single stranded RNA, in contrast to tRNA and DNA, is insoluble in concentrated salt (1 M NaCl) solutions (Jones, 1963).

Details of the methods used to fractionate and characterize our RNA preparations will be described in the next chapter.

MATERIALS AND METHODS USED FOR THE ISOLATION AND CHARACTERIZATION OF RNA FROM CALF LENS CELLS AND RAT LIVER

4.1 INTRODUCTION

The function of messenger or template RNA is to determine the nature of the protein synthesized on ribosomes. It was the aim of this investigation to isolate, compare and characterize RNA preparations with messenger activity from calf lens and from rat liver. At present, however, it is not possible to isolate selectively, messenger RNA from lower or higher organisms. For this reason it was decided to isolate RNA from those subcellular fractions in which the highest messenger activity could be expected on the basis of our present knowledge about RNA and protein synthesis.

In this chapter materials and methods which were used for the islolation and characterization of these RNA preparations with messenger activity, will be described.

4.2 ORIGIN OF THE TISSUES USED

4.2.1 *Lenses*

For the isolation of lenses, eyes of one-day old calves (Dutch pedigree cattle) were used. After the animals were killed in the slaughter-house, the eyes were quickly removed and stored in crushed ice. Thereafter the eyes were immediately transported to the laboratory and the lenses were isolated as soon as possible.

4.2.2 *Livers*

The livers used in our studies were derived from 2 - 3 months old male Wistar albino rats (weight ± 200 g).

The animals were fed with the standard laboratory diet. To prevent disturbance of the cell fractionation due to high concentrations of liver-glycogen, the rats were, where indicated, starved overnight before being killed.

4.3.1 *Polysomes and crude nuclei from calf lens cells*

After isolation of the lenses, the capsules containing the epithelial cells were collected at 0°C. The outer cortex was obtained by punching out a 1–2 mm thick layer from the equator with the aid of a glass trephine. Homogenization of these combined materials was carried out in one volume of ice-cold 0.35 M sucrose in medium B (Medium B consists of 8 mM $MgCl_2$, 25 mM KCl and 50 mM Tris-HCl, pH 7.6). A Teflon homogenizer was used and five strokes at 2,000 revolutions per minute were applied. The homogenate was centrifuged at 12,000 × g for 20 minutes at 2°C in a Sorvall preparative centrifuge.

The pellet was used for the isolation of crude nuclear RNA as will be described in 4.4.2.

The supernatant was used for the isolation of polysomes (Bloemendal et al., 1966b). After addition of deoxycholate (final concentration 0.5%), samples of 15 ml were applied to discontinuous sucrose gradients, each consisting of 2 layers (of 10 ml) of 2 M and 1.5 M sucrose both in Medium B (Bloemendal et al., 1964). The sucrose gradients were then centrifuged at 55,000 × g for 16 hours at 2°C (Spinco rotor 30). The supernatant was sucked off carefully and the pellet, consisting of polyribosomes, was either suspended in a small volume of ice-cold Medium A (Medium A consists of 8 mM $MgCl_2$, 70 mM KCl and 50 mM Tris-HCl, pH 7.6) for the cell-free protein synthesis, or used for the isolation of polysomal RNA as described below (4.4.1).

4.3.2 *Polysomes and microsomal fraction from rat liver*

After starvation overnight the animals were sacrificed by a blow on the head and the livers were quickly removed and placed in a beaker containing 2.5 volumes ice-cold 0.35 M sucrose in Medium B. After homogenization and the first centrifugation as described above for the isolation of lens polysomes, the supernatant was treated in one of two ways.

For the isolation of the liver microsomal fraction and rat liver supernatant, the supernatant (12,000 g) was centrifuged at 105,000 g for 2.5 hours at 0°C.

However, for the isolation of liver polysomes, the supernatant was brought on to a discontinuous sucrose gradient and centrifuged at 75,000 g for 16 hours at 0°C (Bloemendal et al., 1964, 1967; Bont et al., 1967).

After centrifugation the microsomal pellet and the polysomes were suspended in the same media as described above for lens polysomes. The

supernatant, obtained after centrifugation of the homogenate for the isolation of the microsomal fraction, was used as a source of enzymes and tRNA for incubation experiments in the cell-free systems from calf lens or from rat liver.

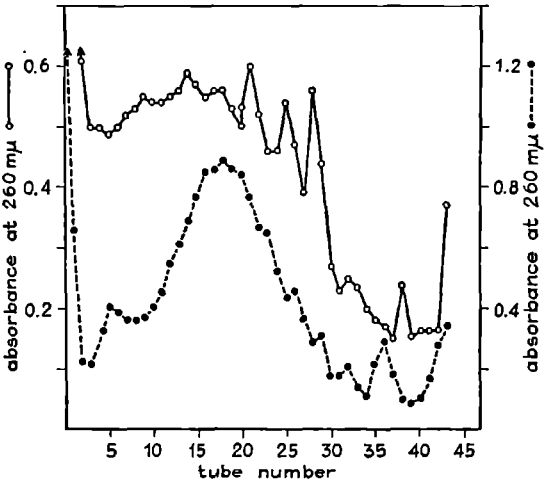

FIGURE 4. Sucrose density gradient centrifugation of lens and liver polysomes. An 8–22% sucrose-gradient containing medium B was used, resting on a cushion of 50% (W/V) sucrose in medium B. Centrifugation was at 25,000 rev/min for 2 hr in rotor SW 25.1.
●– – – –●, Sedimentation pattern of lens polysomes.
o————o, Sedimentation pattern of liver polysomes.

Figure 4 shows the profiles of both lens and rat liver polysomes on a continuous gradient. Only a small amount of monomers is present. The major portion sediments at a much higher rate. Figure 5 is a micrograph of a spread preparation of isolated lens polysomes. Ribosomal aggregates held together by stands are clearly visible (cf. Benedetti et al., 1966a; 1966b).

4.4 PREPARATION OF RNA

4.4.1 *Polysomal RNA from lens cells*

After isolation, the polysomes were suspended in a small volume of ice-cold 100 mM Tris-HCl buffer, pH 7.6, containing 0.5% NDS, 0.01% EDTA, 1% SDS, 0.004% PVS and 5% butanol. The mixture was shaken for

1 minute at 2°C and then an equal volume of ice-cold re-distilled phenol (80% v/v) in 1% SDS, 0.1% 8-hydroxychinoline and 100 mM Tris-HCl, pH 7.6 was added and the shaking continued for 15 minutes at 2°C. After centrifugation at 12,000 × g for 30 minutes at 4°C, the aqueous layer was carefully pipetted off. The phenol layer and interphase were re-extracted with an equal volume of 100 mM Tris-HCl, pH 7.6, and 0.01% EDTA. The RNA in the combined aqueous layers were combined and RNA was precipitated by the addition of 0.1 of its volume of ice-cold 10% NaCl and 2.5 volumes 96% ethanol (-20°C). After thorough mixing and standing overnight in the freezer the RNA precipitate was centrifuged down and dissolved in a small volume of ice-cold 100 mM Tris-HCl, pH 7.6, containing 1% SDS and 0.01% EDTA (final RNA concentration 4 mg/ml). The solution was re-extracted twice with an equal volume of ice-cold phenol (80% v/v), 100 mM Tris-HCl, pH 7.6, containing 1% SDS and 0.1% 8-hydroxychinoline. After the second precipitation of RNA with 0.1 of its volume of 10% NaCl and 2.5 volumes cold ethanol, the RNA was recovered by centrifugation and dissolved in a small volume of ice-cold distilled water (about 10 times the volume of the pellet). An equal volume of ice-cold 20% NaCl was added and the high molecular weight RNA was allowed to precipitate at 4°C overnight. The RNA was collected by centrifugation at 12,000 × g for 20 min. at 2°C and washed twice with 66% ethanol containing 1% NaCl. After centrifugation the RNA pellet was dried with nitrogen gas, dissolved in ice-cold distilled water (final concentration about 4 mg/ml), and stored at -20°C in aliquots of 2–3 mg RNA.

4.4.2 *RNA from the crude nuclear lens pellet*

Immediately after isolation the crude nuclear pellet was suspended in 10 volumes (w/v) of ice-cold 100 mM Tris-HCl, pH 7.6, containing 0.01% EDTA and rapidly homogenized in a Waring blendor for 30 seconds. The homogenate was mixed with an equal volume of ice-cold re-distilled phenol (80% v/v) in 100 mM Tris-HCl, pH 7.6 and 0.01% EDTA for one minute. After thorough mixing in the cold the mixture was placed in a water bath at 39°C while mixing was continued for 30 minutes. Then the mixture was centrifuged at 14,000 × g for 30 minutes at 4°C. The aqueous layer which contained the bulk of the RNA was removed. The interphase was mixed thoroughly with the phenol layer and an amount of 100 mM Tris-HCl, pH 8.3, and 0.01% EDTA equal to the buffer volume used previously, was added. The mixture was stirred at 65°C for 30 minutes and again centrifuged at 14,000 × g for 30 minutes at 4°C. In the initial stage of our study the interphase and phenol layer obtained after the extraction at 39°C were

first mixed and re-extracted twice with an equal volume of 100 mM Tris-HCl, pH 7.6, and 0.01% EDTA at 32°C for 30 minutes. Thereafter the extraction at 65°C and at pH 8.3 was performed as described above. However, in the latter case the recovery of RNA was very low (about 50 µg RNA/1000 lenses). It was for this reason that we decided to re-extract the phenol layer and interphase, obtained after extraction at 39°C (pH 7.6), directly at 65°C (pH 8.3).

RNA from the different aqueous layers was precipitated by addition of 0.1 of its volume of ice-cold 10% NaCl and 2.5 volumes cold ethanol, kept overnight in the freezer (-20°C) and collected by centrifugation. The RNA was dissolved in a small volume (final concentration about 4 mg/ml) of ice-cold 100 mM Tris-HCl, pH 7.6, containing 1% SDS and 0.01% EDTA and the same procedure was repeated as described above for the second phenol extraction of lens polysomal RNA.

As these isolated RNA fractions in some cases appeared to be contaminated with the black pigment of the iris, they were applied to a column of Sephadex G-25 equilibrated with distilled water. The fraction which emerged at the void volume was collected and the RNA present was precipitated by adding 0.1 of its volume of 10% NaCl and 2.5 volumes cold ethanol (-20°C). After standing overnight in the freezer (-20°C) the precipitate was collected by centrifugation. The precipitate was dried with nitrogen gas, dissolved in ice-cold distilled water and stored at -20°C in aliquots of 2–3 mg RNA.

4.4.3 *RNA from polysomes, microsomal fraction and whole rat liver*

The animals were starved overnight before the isolation of microsomal fraction and polysomes, but were fed *ad libitum* with the standard laboratory diet before the isolation of RNA from the whole liver. Isolation of RNA from polysomes and from the microsomal fraction was essentially the same as described above for the isolation of RNA from lens polysomes.

The principal difference between the extraction of RNA from the crude nuclear lens pellet and from the whole rat liver, was that for the latter tissue two extractions at 32°C (pH 7.6) were first performed, prior to the extraction at 65°C (pH 8.3). All further manipulations were the same as described above for the isolation of RNA from the crude nuclear lens pellet. The yield of RNA from whole rat livers extracted at 65°C (pH 8.3), preceded by the extractions at 39°C and at 32°C (pH 7.6), was much higher than the yield of lens RNA from the crude nuclear pellet under the same conditions. All isolated rat liver RNA fractions were dissolved in ice-cold distilled water and stored at -20°C in aliquots of 2–3 mg RNA.

4.4.4 *tRNA from rat liver*

Isolation of tRNA from whole rat liver was performed using a modification of the method described by Brunngraber (1962). The reason for the modification is that tRNA preparations prepared by Brunngraber's method are very often heavily contaminated with DNA, and also the tRNA obtained after column chromatography is not very pure (King, 1967). Rather pure tRNA preparations were obtained by homogenizing rat livers in a mixture of 1.5 volume (w/v) of ice-cold saline (0.14 M NaCl) and an equal volume of re-distilled phenol (80%) in 0.14 M NaCl. Homogenization was performed in a Waring blendor for 1 min. The homogenate was centrifuged for 15 min at 10,000 × g at 4°C. The aqueous layer was pipetted off and NaCl was added to this layer until the concentration was 1 M. Then an equal volume of ice-cold phenol (80% v/v) in 1 M NaCl was added and the same operations repeated as described for the first phenol extraction. To the aqueous layer 2.5 volumes 96% ethanol (-20°C) were added. After standing overnight in the freezer, the precipitate was centrifuged down, dissolved in 1.8 M Tris-HCl, pH 8.0, and incubated for 90 minutes at 37°C (Sarin and Zamecnik, 1964).

The solution was dialyzed against 150 volumes of 100 mM Tris-HCl buffer, pH 7.6, the buffer was changed twice, and then directly applied to a DEAE-Sephadex A-50 column, equilibrated with the same buffer. After application of the RNA solution, the column was washed with 100 mM Tris-HCl, pH 7.6 until the extinction of the eluate at 260 mμ was lower than 0.1/cm. Then a linear salt-gradient (ranging from 0.1 M to 1 M NaCl in 100 mM Tris-HCl, pH 7.6) was started. The tRNA was eluted from the column at NaCl concentrations ranging from 0.45 M to 0.7 M. To the pooled tRNA fractions 2.5 volumes 96% ethanol (-20°C) was added. The resulting tRNA precipitate was then treated in the same manner as described for the lens RNA preparations. The isolated tRNA preparations were routinely tested on polyacrylamide gels. As can be seen in figure 6, the isolated tRNA was free from other RNA contaminations.

4.4.5 *Tobacco mosaic virus RNA*

Tobacco mosaic virus was the generous gift of Dr. L. van Griensven (Lab. voor Virologie, Landbouwhogeschool, Wageningen, The Netherlands). RNA was isolated according to the method of Fraenkel – Conrat (1966), the isolation being performed in the presence of bentonite (Brownhill et al., 1959).

4.4.6 *Turnip yellow mosaic virus RNA*

TYMV-RNA was the generous gift of Dr. M. Hierowski, Poznan, Poland (a guest investigator in our laboratory).

4.4.7 *Rauscher virus RNA*

Rauscher virus was isolated by Dr. J. H. Burghouts (Laboratorium voor Biochemie, Nijmegen).

RNA was extracted according to the same procedure as described above for the isolation of RNA from lens polysomes.

4.5 SUCROSE DENSITY GRADIENT ANALYSIS

Sucrose has been generally used for the preparation of density gradients to separate RNA molecules of different molecular weight. Linear gradients of sucrose (5–20%) in 10 mM sodium acetate buffer, pH 5.1 containing 100 mM NaCl, were prepared using a device described by Britten and Roberts (1960). RNA (2–3 mg in 1 ml buffer without sucrose) was carefully layered on to 28 ml of the gradient and centrifuged for 11 hours at 25,000 rpm at 2°C in a swing-out rotor, type 25.1 of a preparative ultracentrifuge (Spinco L-50). The rotor was allowed to decelerate slowly without use of the brake. After the run the tubes containing the sucrose gradients were transferred to a Perspex holder. The bottoms of the tubes were punctured with a needle which was introduced through the outlet and 10 drop fractions were collected. From each fraction 100 µl was diluted with 1 ml distilled water and the absorbance at 260 mµ was measured with the aid of a Zeiss-Spectrophotometer (type PMQ II). By using the approximation of Martin and Ames (1961) and the values of 18S and 28S for the ribosomal RNA peaks, the sedimentation coefficient of other RNA fractions in the sedimentation pattern could be estimated. The remaining portions of the gradient were combined in 8 to 12 groups and the corresponding regions of three separate gradients were pooled. To each fraction 2.5 volumes ethanol (-20°C) was added. After standing overnight in the freezer (-20°C) the precipitate was centrifuged down, dried with nitrogen gas and dissolved in an appropriate buffer or in distilled water. Instead of puncturing the tube and collecting drops by hand, an alternative method was used in which the gradient was pushed out by pumping a 40% sucrose solution through a fine needle reaching to the bottom of the tube. The gradient outlet at the top was connected with a micro flow-cell from a Gilford Spectrophotometer (type 2000). The extinction was continuously measured at 260 mµ and

registered on a chart of the Gilford recording system. Fractions of 10 droplets were collected with the aid of an LKB fraction collector.

Sucrose density gradient analysis of incubation mixtures or of polysomes were performed as indicated in the legends to the figures.

4.6 ELECTROPHORESIS OF RNA FROM CALF LENS AND RAT LIVER ON POLYACRYLAMIDE GELS

Polyacrylamide gel electrophoresis of high molecular weight RNA was performed according to Loening (1967). 2.2% polyacrylamide gels were prepared as described by Bishop et al. (1967). Gels were allowed to polymerize in plexiglass tubes (6 × 0.6 cm) which were supported by a dialysis membrane stretched across the lower end. The polymerization time was about 20 minutes.

A pre-electrophoretic run was performed for 1 hour to remove catalyst and other unpolymerised chemicals. After pre-electrophoresis the RNA sample (40–70 µg), dissolved in 20–60 µl of the electrophoresis buffer (40 mM Tris – 60 mM sodium acetate – 1 mM EDTA adjusted to pH 7.2 with glacial acetic acid), was then layered on to the gels with the aid of 5% sucrose (w/v) and electrophoresis continued at room temperature for 90 min (5 mA per gel). Where indicated polymerization and electrophoresis was performed in the presence of 6 M urea. After electrophoresis the gels were stained with 0.2% toluidine blue 0 in 10% acetic acid for at least 4 hours. The gels were de-stained by electrophoresis perpendicular to the length of the gels (20 mA per gel). De-staining was accomplished within 30 minutes.

Staining with pyronin Y (Richards et al., 1965) or with acridine orange (Gould, 1966a, 1966b) appeared to be less satisfactory than toluidine blue 0. As the gels are very soft they were handled with the aid of a plexiglass tube (inner diameter was equal to the diameter of the gel) on which a rubber teat was mounted. After being de-stained the gels were transferred to glass tubes which had an inner diameter nearly equal to the diameter of the gel. It is necessary to store the gels in this way, otherwise comparison of the electrophoretic patterns of different RNA preparations is very difficult. It should be mentioned that the presence of polyvinyl sulphate strongly disturbs the staining process as the upper part of the gel is obscured by a very strong pink colour.

Attempts to fractionate RNA on a preparative scale, using a "Poly Prep" preparative gel electrophoresis apparatus (Buchler Instr. Inc.) have not been successful. After some hours of electrophoresis the gel loosened from the outer wall of the apparatus, causing a drop in the current. Finally the

current in the gel ceased. Attempts to overcome this difficulty by varying the temperature during electrophoresis, changing the buffer composition or adding agarose to the gel (Peacock and Dingman, 1968), all failed. Similar observations have been made by other investigators (Chrambach, 1968).

4.7 DETERMINATION OF THE BASE COMPOSITION OF RNA

Before the base composition of RNA can be determined, the RNA fraction in question must first be hydrolyzed.
Hydrolysis of RNA was performed in 0.3 N KOH for 18 hours at 37°C (Marrian et al., 1951). After cooling in ice, the solution was neutralized to pH 4 with 70% perchloric acid. The precipitate was centrifuged down and the supernatant was used for the electrophoretic experiments. Electrophoretic separation of the ribonucleotides was carried out according to the method of Markham and Smith (1955). Electrophoresis was performed on sheets of Whatman paper No. 1 (45 × 15 cm) in 0.05 M ammonium formate buffer, pH 3.5 for 2.5 hours at 2,000 volt. Samples of about 100 µg of ribonucleotides were applied per centimetre width of origin. After the electrophoretic run the paper was air dried. The nucleotides were localised with the aid of a Miniralight short wave (200 – 280 mµ) Transilluminator (U.V. Prod. Inc. San Gabriel, California). The spots were marked and cut out. The nucleotides were eluted overnight with 2 ml of 0.01 N HCl at 37°C and assayed by their absorbance at optimum wavelengths. Blanks were cut out equal in area to the spots and at equal distances from the starting line and eluted in the same manner as the corresponding nucleotides.

4.8 CULTIVATION OF *ESCHERICHIA COLI* B

The strain B of *E. coli* was cultivated on an agar slant culture containing Trypton Soya Agar (Oxoid Division of OXO Ltd., London). For the cultivation of large amounts of bacteria a two step growth procedure was used.

4.8.1 *The first step in the cultivation of E. coli* B

A sterile nutrient broth – glucose solution (250 ml in a 500 ml Erlenmeyer flask) was inoculated with *E. coli* B grown on the agar slant. Thereafter the flasks were incubated overnight at 30°C. The medium contained 8 g of nutrient broth (Difco and Co., Detroit, Michigan) and 5 g of glucose per litre of tap water. The glucose solution (60%) was autoclaved separately and was added to the sterile nutrient broth solution after being cooled.

4.8.2 *The second step in the cultivation of E. coli* B

While the culture was still in the logarithmic growth phase it was added to 12 litres of medium (at 37°C) with the same composition as mentioned above. The cells were then further cultivated at 37°C while the medium was aerated vigorously. To prevent foam formation an antifoam (Sigma Anti foam spray stock No. AFS) was added. Cell growth was followed carefully by measuring the turbidity of culture samples taken at different intervals. The turbidity was measured at 540 mμ with the aid of a Zeiss PMQ II spectrophotometer. Aeration was stopped when a turbidity of approximately 0.5 was reached compared to a sterile nutrient broth glucose solution. The cultivation time was then about 2 hours and the culture was still in the early logarithmic phase. The culture was cooled by the addition of crushed ice and the cells were harvested by centrifugation at 2°C. The packed cells were washed three times (at 0°C) by rapid suspension in 150 ml ice-cold 10 mM Tris-HCl buffer, pH 7.8, containing 14 mM magnesium acetate and 60 mM potassium chloride. The suspension was quickly centrifuged, the supernate solutions decanted and the pellets were well drained. The yield was 0.5 – 0.6 g wet weight of packed cells, per litre of medium. Washed packed cells were frozen quickly and stored at -20°C.

A third step was sometimes performed after the second cultivation step. The culture of the second step was then transferred to a further 40 litres of the nutrient broth-glucose medium. The cultivation was then performed in the same manner as described for the second step culture. Thereafter the cells were harvested by means of a water-cooled Sharpless continuous-flow centrifuge. The method for washing and storing the packed cells was the same as that previously described.

4.9 PREPARATION OF THE CELL-FREE EXTRACT

The procedure used was a modification of the method described by Nirenberg and Matthaei (1961). All operations, unless otherwise specified, were carried out in a cold room at 2°C.

About 5 g of packed cells (-20°C) were transferred to a large pre-chilled, unglazed, porcelain mortar and 5 g pre-chilled aluminium oxyde (Alcoa A-305) was added. The cells were ground vigorously with a pestle until a thick paste was obtained. When the cells were broken, an additional 5 g of aluminium oxide was added. After about 10 min of grinding, 10 ml of 10 mM Tris-HCl buffer, pH 7.8, containing 14 mM magnesium acetate, 60 mM potassium chloride and 6 mM 2-mercaptoethanol was added, and the paste was suspended by gentle stirring. Alumina, intact cells and debris were removed by centrifugation for 20 min at 20,000 × g at 2°C. The

supernatant was decanted and the pellet discarded. To prevent additional synthesis of messenger RNA 5 μg of pancreatic DNase was added and gently mixed to each ml of crude extract. After incubation for 10 min at 0°C, the extract was centrifuged twice for 30 min at 30,000 × g at 2°C. The supernatant solution was removed by aspiration to within 1 cm of the pellet and referred as the S-30 fraction.

To reduce the concentration of endogeneous messenger RNA, the S-30 fraction was preincubated with all the factors necessary for protein synthesis. To 10 ml S-30, 1 ml of 500 mM Tris-HCl pH 7.8, 0.2 ml of 158 mM magnesium-acetate, 0.4 ml of 8.1 mM ATP and 2.0 mM GTP (Na-salts), 1.2 ml of 3 mM PEP (Na-salt), 4 μl of 2-mercaptoethanol and 65 μmoles from each of 20 L-amino acids, were added.

After a preincubation period of 80 min at 37°C, the reaction mixture was cooled to 0°C and centrifuged to remove denaturated protein. To remove endogenous amino acids and low molecular weight degradation products of RNA and DNA the supernatant was subjected to gel filtration on a column of Sephadex G-25 equilibrated with 10 mM Tris-HCl buffer, pH 7.8, containing 14 mM magnesium acetate and 60 mM HN_4Cl. The fractions emerging at the void volume were collected, divided into 1 ml portions, and rapidly frozen in a dry ice-acetone mixture. These fractions, referred to as iS-30 fractions, were stored at -20°C. This extract, which contained all factors necessary for protein synthesis, could be used without further purification to assay RNA fractions for messenger activity. *E. coli* ribosomes were isolated according to Voorma (1965).

4.10 ASSAY FOR MESSENGER ACTIVITY

In vitro polypeptide synthesis was performed in reaction mixtures of 250 μl, which were composed of the components as summarized in table 6.

After incubation at 37°C for 45 minutes (unless otherwise specified) the reactions were stopped by addition of 2 ml of 10% TCA. After centrifugation the precipitate was re-suspended in 3 ml of 5% TCA, boiled for 15 min at 90° (to hydrolyse aminoacyl-tRNA) poured on to Millipore filters (diameter 25 mm, pore size 0.45 μ) and washed several times with cold 5% TCA. After drying at 65°C for 30 min, the samples were either counted on a thin window gas flow counter (Nuclear Company Chicago, efficiency 20%) or the filter containing the dried precipitate was placed in a glass counting vial containing 10 ml scintillation fluid composed of 0.3% PPO and 0.02% POPOP in toluene. Radioactivity was measured in a Packard Tri-carb liquid scintillation spectrometer (Model 4322), with an efficiency of 80%. All assays were performed in duplicate.

TABLE 6. Composition of the reaction mixture for *in vitro* polypeptide synthesis

component	volume
cell-free extract of *E. coli* B (in 10 mM Tris-HCl, pH 7.8; 14 mM magnesium acetate; 60 mM ammonium chloride; 6 mM 2-mercaptoethanol).	0.1 ml
PEP (75 mM in water)	0.025 ml
Nucleoside triphosphates (20 mM ATP; 2.5 mM GTP in 10 mM Tris-HCl, pH 7.8; 192 mM NH_4Cl; 84 mM magnesium acetate).	0.025 ml
Pyruvate kinase (10 mg per ml).	0.0004 ml
tRNA *E. coli* B (5 mg/ml water).	0.01 ml
^{12}C-amino acids minus the appropriate radioactive amino acid(s) (10 nmoles of each).	0.004 ml
^{14}C-amino acid(s) (amount and specific activity as indicated).	0.005 ml
RNA (in 10 mM Tris-HCl, pH 7.8; 60 mM ammonium chloride).	0.05 ml
water	to a final volume of 0.25 ml

4.11 QUALITATIVE AND QUANTITATIVE DETERMINATIONS

Absorption spectra of ribonucleotides were measured in a Beckman-DB spectrophotometer. RNA and ribonucleotide concentrations were measured by their absorbance at optimal wave lengths with the aid of a Zeiss-spectrophotometer (type PMQ II). RNA concentrations were calculated from the absorbance at 260 mμ, assuming that RNA at a concentration of 1 mg per ml had an extinction of 25.

DNA was determined by the diphenylamine method, as described by Burton (1956). Calf thymus DNA was used as a standard.

Protein was determined according to the method of Lowry et al. (1951). Bovine serum albumin was used as a standard.

Acrylamide – Union Chimique Belge
ADP (tri-natrium salz) – C F Boehringer und Soehne
AMP (di-natrium salz) – C F Boehringer und Soehne
Antifoam Spray (Stock no AFS) – Sigma
ATP (kristall, di-natrium salz) – C F Boehringer und Soehne
Bovine Albumin – Nutritional Biochemicals
Bentonite (technical) – The British Drug Houses Ltd
^{12}C-Amino acids – Sigma
^{14}C-Amino acids – The Radiochemical Centre, Amersham
CMP (di-natrium salz) – C F Boehringer und Soehne
DEAE-Sephadex A-50 – Pharmacia
Deoxyribonuclease (EC 3 1 4 5, RNase free) – Sigma
DNA – Miles Laboratories inc
Dowex 1×2 – Fluka
E coli tRNA (Strain B-Stripped) – General Biochemicals
Folin-Ciocalteus phenol reagent – E Merck A G
GDP (di-natrium salz) – C F Boehringer und Soehne
GMP (di-natrium salz) – C F Boehringer und Soehne
GTP (tri-lithium salz) – C F Boehringer und Soehne
N-Hydroxysuccinimide – K&K Laboratories inc
8-Hydroxyquinoline G R – E Merck AG
2-Mercaptoethanol – Koch Light Laboratories Ltd
NDS (purified sodium salt) – The British Drug Houses Ltd
N,N′ – methylen-bis acrylamide – Schuchardt, Munchen
Nutrient Broth – Difco Laboratories
PCA – E Merck AG
Phenol – E Merck AG
Phosphoenol pyruvic acid (tri-sodium salt-hydrate) – Sigma
Polyuridylic acid (ammonium salt) – Miles Laboratories inc
POPOP (scintillation grade) – Packard Instrument Company inc
PPO (FP 70–72°C for scintillation measurings) – E Merck AG
Puromycin (dihydrochlorid) – Nutritional Biochemicals Corporation.
PVS (potassium salt) – General Biochemicals
Pyruvate kinase (EC 2 7 1 4) – C F Boehringer und Soehne
Ribonuclease p a (EC 2 7 7 16) – C F Boehringer und Soehne
Sephadex G-25 or G-50 – Pharmacia
SDS – Sigma
Sucrose (from sugar cane) – BDH Chemicals Ltd
Toluidene blue 0 – E Merck AG

CHARACTERISTICS OF THE TEST SYSTEM

5.1 INTRODUCTION

In chapter 2 it was stressed that the stimulation of amino acid incorporation into protein is the main criterion for the detection of messenger RNA. The cell-free system used for detecting this activity was prepared from *E. coli* B.

There were two reasons for using this system. Firstly the cell-free system from *E. coli* has been quite well characterized and secondly, the synthesis of several non-homologous proteins, coded by exogenous messenger RNA, has been detected (see 2.8).

In order to study the isolated RNA preparations with respect to messenger activity it is of the utmost importance that the endogenous messenger activity is as low as possible. For this reason the cell-free extract is preincubated under circumstances of optimal protein synthesis, in order to achieve an effective breakdown of endogenous messenger RNA.

As it is important to find out the optimal conditions for protein synthesis, directed by endogenous messenger RNA as well as by exogenous messenger RNA, the results of such an investigations are described in this chapter.

5.2 AMINO ACID INCORPORATION INTO PROTEIN IN THE NON-PREINCUBATED CELL-FREE EXTRACT OF *E. COLI*

5.2.1 *Magnesium concentration dependence*

It is generally known that the amino acid incorporation is strongly dependent on the presence of magnesium ions. For instance enzymes such as aminoacyl synthetases are dependent on magnesium ions for their catalytic effect (Bublitz, 1966; George and Meister, 1967; Hahn and Brown, 1967; Svensson, 1967a; 1967b). Magnesium ions are also required in the reactions and interactions involved in protein synthesis. For example, they are not only required for binding of messenger RNA to ribosomes (Gros et al., 1961; Brenner et al., 1961; Barondes and Nirenberg, 1962c; Gilbert, 1963; Okamoto et al., 1963; Voorma et al., 1965; Dahlberg and Haselkorn, 1967a; 1967b), but also for the interaction of tRNA with a specific site of the ribosome (chapter 2 and Cannon et al., 1963).

Magnesium ions are also required for the integrity of ribosomes When the magnesium ions are at a concentration of 10 mM, the ribosomes are in the 70S form, but at a magnesium concentration of 0 1 mM, the 70S ribosomes dissociate into their 50S and 30S subunits (Tissières et al , 1959, Bowen et al , 1961, Green and Hall, 1961; Schlessinger and Gros, 1963, Petermann, 1964) Removal of magnesium ions by addition of EDTA results in the conversion of 50S ribosomal subunits to 21S particles, and 30S ribosomal subunits to 16S particles (Gesteland, 1966)

From these observations it is clear that the integrity of ribosomes, as well as the interaction of nucleic acids with ribosomes, is strongly dependent on the magnesium ion concentration

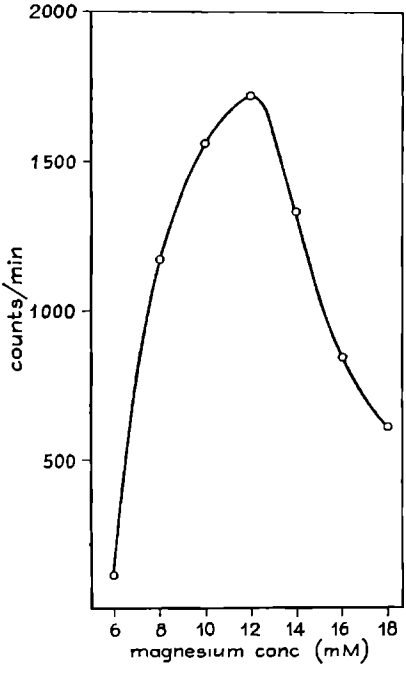

FIGURE 7 Magnesium concentration dependence of the endogenous mRNA directed amino acid incorporation in the non-DNase treated cell-free extract of *E coli*

The reaction mixture contained the components as described in table 6, except that the magnesium concentration varied as indicated Incubations were performed in the presence of 12 nmoles DL-[l-^{14}C]-leucine (specific activity 34 mCi/mmole) and 2nmoles unlabelled L-leucine The reactions were terminated after 60 minutes of incubation (37°C) Samples were counted in a thin window gas flow counter

Optimal incorporation of amino acids into protein in the presence of endogenous messenger RNA was found at a magnesium concentration of 12mM (figure 7). This optimum is of the same order of magnitude as that found by other investigators working with the same and various other strains of *E. coli* (Tissières et al., 1960). To find optimal effects these experiments were performed with extracts to which no extra DNase was added as the depletion of DNase permits the synthesis of new endogenous messenger RNA (Capecchi, 1966a).

5.2.2 *Kinetics of the amino acid incorporation in the non-preincubated cell-free extract*

Incorporation of amino acids is strongly dependent on the duration of incubation. The main reason for the amino acid incorporation stopping after a definite incubation time, is a rapid degradation of messenger RNA (Barondes and Nirenberg, 1962b; Spyrides and Lipmann, 1962; Sarkar and Dürwald, 1966) and in most cases no new RNA synthesis occurs. The kinetic experiments revealed that the amino acid incorporation into protein in the DNase treated non-preincubated system reached a plateau after 60–80 minutes of incubation (figure 8). Therefore the cell-free extract was preincubated for 80 minutes at 37 °C.

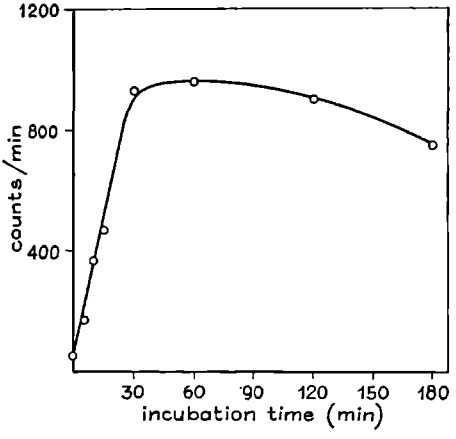

FIGURE 8. Kinetics of amino acid incorporation in the non-preincubated cell-free extract of *E. coli*.

The reaction mixture contained the components as described in table 6. Incubations were performed in the presence of 10 nmoles DL-[1-^{14}C]-leucine (specific activity 55.2 mCi/mmole) and 3 µmoles of magnesium acetate. Samples were counted in a thin window gas flow counter.

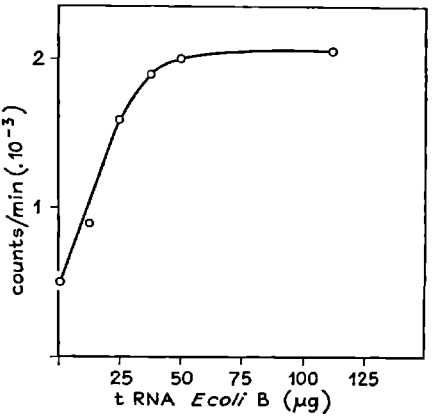

FIGURE 9. Dependence on tRNA of the endogenous mRNA directed amino acid incorporation.

The reaction mixture contained the components as described in table 6, except that the tRNA concentration varied as indicated Incubations were performed in the presence of 10 nmoles DL-[l-^{14}C]-leucine (specific activity 55 2 mCi/mmole) and 3 µmoles magnesium acetate Samples were counted in a thin window gas flow counter.

5 2 3 *tRNA dependence of the endogenous mRNA directed amino acid incorporation*

Protein synthesis is strongly dependent on the presence of tRNA. As it was important to know that the amino acid incorporation into protein was occuring under optimal conditions, the effect of addition of extra tRNA (from *E. coli* B) on the endogenous mRNA directed leucine incorporation was investigated.

As is shown in figure 9 the incorporation of leucine into protein by the S-30 fraction was stimulated by the addition of purified *E coli* tRNA, despite the fact that in the S-30 fraction tRNA is already present. Maximal stimulation was obtained with approximately 200 µg tRNA per ml incubation mixture. Therefore extra tRNA was added at this concentration to all reaction mixtures unless otherwise specified.

5.3 AMINO ACID INCORPORATION INTO PROTEIN IN THE PREINCUBATED CELL FREE EXTRACT OF *E COLI*

5 3.1 *Effect of dialysis and gel filtration of the preincubated extract on the poly U directed phenylalanine incorporation*

Nirenberg and Matthaei (1961) as well as several other investigators

have applied dialysis after preincubation of the cell-free extract in order to remove endogenous amino acids and other low molecular weight material which may decrease or block the incorporation of amino acids (Nirenberg, 1963). An analogous dialysis-step was also performed in the present study. It frequently happened, however, that the poly U directed phenylalanine incorporation by the dialysed extract was very low compared with the incorporating activity of the non-dialysed extract. In contrast, gel filtration of the preincubated extract on a Sephadex G-25 column resulted in no loss of incorporating activity (table 7). This result may be explained

TABLE 7. Effect of dialysis or gel filtration on Sephadex G-25 of the preincubated extract on the poly U directed phenylalanine incorporation.

	Addition	Amino acid incorporation counts/min/mg protein	Relative stimulation
DIALYSIS			
	None	90	—
	None, deproteinized at zero time	62	—
	40 µg poly U	15,253	170–fold
	40 µg poly U	15,253	170–fold
GEL-FILTRATION			
	None	78	—
	None, deproteinized at zero time	58	—
	40 µg poly U	20,857	267–fold

The reaction mixture contained the components described in table 6. Incubations were performed in the presence of 8 nmoles unlabelled L-phenylalanine, 0.4 nmoles L-^{14}C-phenylalanine (uniformly labelled; specific activity 459 mCi/mmole) and 4.5 µmoles magnesium acetate. Samples were counted in a thin window gas flow counter.

by assuming that during the course of dialysis (usually 24 hours) there is a loss of material (caused by denaturation or breakdown) which is indispensable for the amino acid incorporating activity of the cell-free extract. On the other hand, column chromatography on Sephadex G-25 is a much quicker procedure (4 hours) and no loss of high molecular weight material occurs. The fraction emerging with the void volume was (F1, figure 10) collected and used for the incubation experiments.

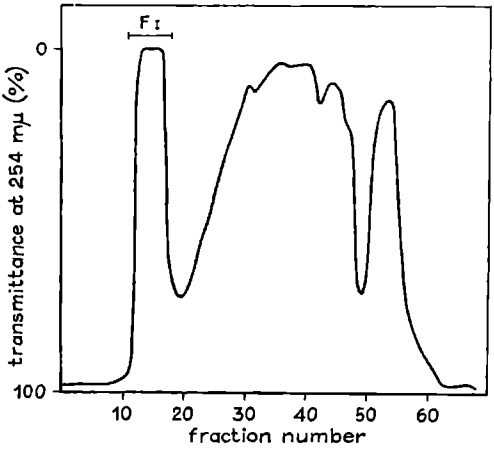

FIGURE 10. Chromatography of the preincubated extract of *E. coli* on Sephadex G-25.

The preincubated extract (15 ml) was applied to a Sephadex G-25 column (50×3cm) equilibrated with 10 mM Tris-HCl buffer, pH 7.8; containing 60 mM NH_4Cl, 14 mM magnesium acetate and 6 mM 2-mercaptoethanol. Elution was accomplished at 2°C at 20 ml/hr. The effluent was monitored at 254 mμ using an LKB Uvicord absorptiometer. The fraction volume was 5 ml.

5.3.2 *Magnesium concentration dependence of the poly U directed phenylalanine incorporation*

As it was a prerequisite to have a well-defined extract for investigations of the messenger activity of RNA preparations, we chose poly U directed phenylalanine incorporation as the measure of extract efficiency. For this reason it was important to find the optimal conditions for poly U directed phenylalanine incorporation.

Optimal incorporation of phenylalanine into polypeptides in the presence of poly U was achieved between 18–20 mM magnesium (fig. 11), which is in accordance with the results obtained by other investigators (Matthaei et al., 1967; Cathey et al., 1967).

Surprisingly, the intracellular concentration of bound and free magnesium ions in *E. coli* is much lower than the concentration required here for optimal poly U directed phenylalanine incorporation (Hurwitz et al., 1967). From experiments described by Lucas-Lenard and Lipmann (1967), it became clear that the finding of a high magnesium concentration for the optimal poly U directed phenylalanine incorporation is the consequence of the

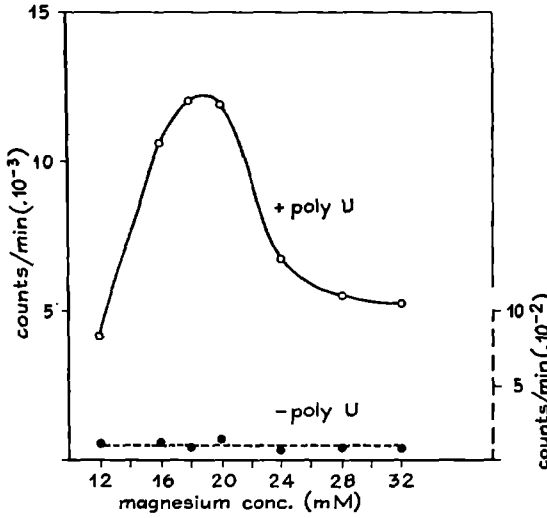

FIGURE 11. Effect of the magnesium concentration on the poly U directed phenylalanine incorporation.

The reaction mixture contained the components as described in table 6, except that the magnesium concentration was varied as indicated. Incubations were performed in the presence of 40 µg poly U, 7 nmoles DL-^{14}C-phenylalanine (specific activity 44.2 mCi/mmole) and 6 nmoles unlabelled L-phenylalanine. Samples were counted in a thin window gas flow counter.

absence in poly U of an initiator codon. Efficient functioning of the initiation mechanism is only possible at low magnesium ion concentrations, as only under such conditions is there selective binding of both ribosomes to messenger RNA, and tRNA to the "peptidyl-site". These investigators found that the addition of acetylphenylalanyl-tRNA to the cell-free system has a drastic effect on the optimal magnesium concentration for poly U directed phenylalanine incorporation.

The code-word UUU is not, however, an initiator codon and the occurrence of acetylphenylalanyl-tRNA has never been detected. Hence one has to be very careful when interpreting these results in terms of chain initiation (see also 2.4.2).

5.3.3 *Kinetics of the poly U directed phenylalanine incorporation*

Kinetic experiments revealed that the poly U directed phenylalanine incorporation proceeded at a linear rate for approximately 30 minutes.

After that time the incorporation rate gradually decreased (figure 12). Even after 80 minutes no definite plateau for the phenylalanine incorporation was reached. Addition, after 45 minutes of incubation, of fresh poly U to the incubation mixture resulted in a resumption of the phenylalanine incorporation (Table 8). Stepwise addition of poly U to the incubation mixture also resulted in a higher phenylalanine incorporation than a single addition of poly U. These results indicate a continuous breakdown of poly U during the course of incubation due to endogenous RNase activity (Barondes and Nirenberg, 1962b; Spyrides and Lipmann, 1962). In addition it is possible that inactivation occurs due to the gradual release of ribosome bound RNase activity (cf. Wade and Robinson, 1965; Bosch et al., 1966).

5.3.4 *tRNA dependence of the poly U directed phenylalanine incorporation*

The poly U directed phenylalanine incorporation at various concentrations of tRNA is illustrated in figure 13. About 800 µg of tRNA per ml incubation

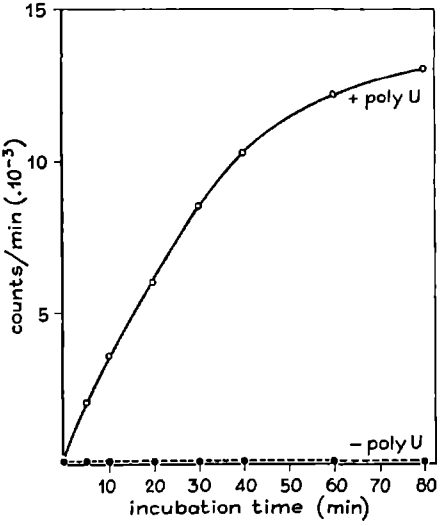

FIGURE 12. Kinetics of the poly U directed phenylalanine incorporation.

The reaction mixture contained the components as described in table 6. Incubations were performed in the presence of 40 µg poly U, 8 nmoles unlabelled L-phenylalanine, 0.4 nmoles L-^{14}C-phenylalanine (uniformly labelled; specific activity 495 mCi/mM) and 4.5 µmoles magnesium acetate. Samples were counted in a thin window gas flow counter.

TABLE 8. Effect of stepwise addition of poly U on phenylalanine incorporation in the preincubated cell-free extract of *E. coli*.

Addition	Incubation time (minutes)	Amino acid incorporation counts/min/mg protein	Relative stimulation
None	45	55	—
None, deproteinized at zero time	0	43	—
40 µg poly U, added at zero time	45	8,233	150–fold
40 µg poly U, added stepwise after 0', 15' and 30 min of incubation	45	10,997	198–fold
40 µg poly U, added after 30 min of incubation	75	6,308	133–fold
None, complete system	90	78	1.5–fold
40 µg poly U, added at zero time	90	9,128	166–fold
80 µg poly U, added stepwise after 0' and 45 min of incubation	90	11,983	218–fold

The reaction mixture contained the components as described in table 6.
Incubations were performed in the presence of 8 nmoles unlabelled L-phenylalanine, 0.20 nmoles L-^{14}C-phenylalanine (uniformly labelled; specific activity 504 mCi/mmole) and 4.5 µmoles magnesium acetate. Samples were counted in a thin window gas flow counter.

mixture were required for optimal amino acid incorporation. Sometimes an even higher amount of tRNA was found to be necessary. The tRNA concentration necessary for optimal poly U directed phenylalanine incorporation is much higher than that necessary for optimal incorporation directed by natural messenger RNA, (figure 9). These differences may be related to a difference in the coding properties of the two species of messenger. Poly U codes only for one amino acid while the natural messenger RNA codes for all amino acids. Therefore it is plausible that poly U requires a higher concentration of phenylalanyl-tRNA, than natural messenger RNA does.

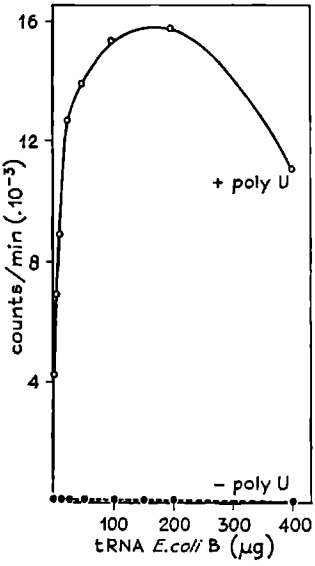

FIGURE 13. Dependence on tRNA of the poly U directed phenylalanine incorporation.

The reaction mixture contained the components as described in table 6, except that the tRNA concentration varied as indicated. Incubations were performed in the presence of 40 µg poly U, 8 nmoles unlabelled L-phenylalanine, 0.4 nmoles L-^{14}C-phenylalanine (uniformly labelled; specific activity 495 mCi/mmole). Samples were counted on a thin window gas flow counter.

The inhibitory effect observed at high tRNA concentrations may be related to the presence of a modified tRNA, which has been shown to inhibit the phenylalanine incorporation (Abdel-Aziz Mostafa et al., 1967; Culp et al., 1968).

5.3.5 *Dependence on the poly U concentration of the phenylalanine incorporation*

For several reasons it was necessary to know at which poly U concentration the maximal phenylalanine incorporation could be obtained.

The incorporation of phenylalanine into polypeptides at different concentrations of poly U is illustrated in figure 14. About 200 µg of poly U per ml incubation mixture were required for an optimal phenylalanine incorporation. Sometimes, however, the maximal phenylalanine incorpo-

ration was only reached at a higher poly U concentration, and at other times high poly U concentrations resulted in a slight inhibition of the phenylalanine incorporation.

An explanation for these discrepancies may be found in the differences in the prepared extracts (nuclease content) or in the differences of the average molecular weights of the poly U preparations used for our study (Matthaei et al., 1962, Martin and Ames, 1962; Jones et al, 1964). The inhibition of the phenylalanine incorporation at high poly U concentration may possible also be related to a lowering by poly U of the actual magnesium concentration. Lowering of the magnesium concentration - due to complex formation - is coupled with a lowering of the incorporating activity (figure 11).

5.3.6 *Binding of poly U to ribosomes*

The first step in the reactions leading up to the formation of the first peptide bond is the binding of messenger RNA to ribosomes (see 2.4). Magnesium ions are required for this binding. It has been suggested that ionic bridges exist between the phosphate groups of messenger RNA and those of ribosomal RNA. It has been demonstrated that magnesium can be replaced by other divalent cations (calcium and manganese) and spermidine

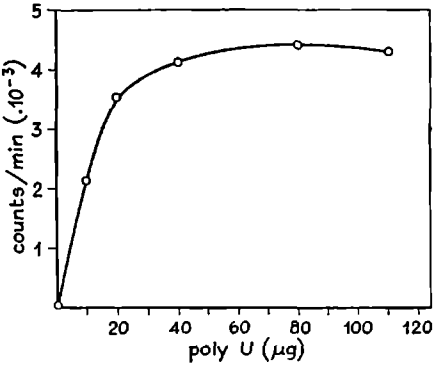

FIGURE 14. Dependence on the poly U concentration of the phenylalanine incorporation.

The reaction mixture contained the components as described in table 6, except that the poly U concentration varied as indicated. Incubations were performed in the presence of 10 nmoles unlabelled L-phenylalanine, 0 26 nmoles L-^{14}C-phenylalanine (uniformly labelled; specific activity 504 mCi/mmoles). Samples were counted in a thin window gas flow counter.

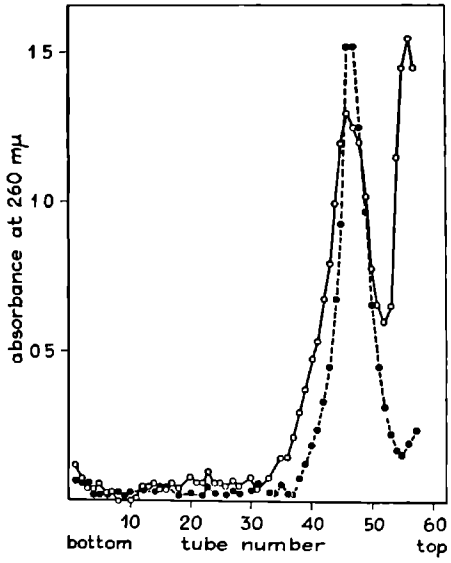

FIGURE 15 Sedimentation pattern of *E coli* ribosomes in the presence and absence of poly U.

E. coli ribosomes (100 μg) were incubated (10 min at 0°C) with and without poly U (70 μg) in 0 5 ml Tris-HCl buffer, pH 7 8, containing 60 mM NH_4Cl and 18 mM magnesium acetate After incubation the mixture was layered on a sucrose gradient (18–22%) containing 18 mM magnesium acetate, 60 mM NH_4Cl, buffered to pH 7 8 with 10 mM Tris-HCl and centrifuged for 2 hours at 25,000 rpm in rotor SW 25 1

●– – – –● Sedimentation pattern of ribosomes incubated in the absence of poly U

o————o Sedimentation pattern of ribosomes incubated in the presence of poly U

(Moore, 1966a; Moore and Asano, 1966, Gordon, 1966 and see also Devi et al , 1961) Selective chemical modifications have led to the proposal that messenger RNA binding results from hydrogen bond formation between the amino-groups of ribosomal RNA and the phosphate groups of messenger RNA (Moore, 1966b).

Figure 15 illustrates the binding of poly U to *E coli* ribosomes Ribosomes (prepared according to Voorma, 1965) were incubated with poly U at 0°C for 10 minutes. After incubation, the mixture was analysed by sucrose density gradient centrifugation (8–22%). A small shift of the material absorbing at 260 mμ towards the bottom of the tube was observed This

shift is strongly dependent on the length of the poly U molecules, since longer molecules bind more ribosomes and consequently a higher sedimentation value is obtained. Similar results have been described by other investigators (Takanami et al., 1963; 1964; Gilbert, 1963; Spyrides and Lipmann, 1962; Barondes and Nirenberg, 1962c).

5.4 CONCLUSIONS

The non-preincubated extract had a maximal amino acid incorporating activity at a magnesium concentration of about 12 mM (figure 7).

Maximal endogenous mRNA directed amino acid incorporation was reached after 60–80 minutes of incubation (figure 8).

The preincubated extract was more active in the poly U directed phenylalanine incorporation after gel filtration on Sephadex G-25 than after dialysis (table 7).

Maximal poly U directed phenylalanine incorporation was obtained at a magnesium concentration of 18 mM and after at least 45 minutes of incubation (figures 11 and 12).

The tRNA dependence of the endogenous messenger RNA directed amino acid incorporation differed strongly from the poly U directed phenylalanine incorporation (figures 9 and 13).

Breakdown of poly U during incubation could be demonstrated, since stepwise addition of poly U to the incubation mixture resulted in higher phenylalanine incorporation than a single addition of the total amount (table 8).

The other characteristics of the system described in this chapter are comparable with those described by other investigators using the same and various other strains of *E. coli*.

These results led us to adopt this incubation system to study the messenger activity of RNA preparations as described in chapter 6.

MESSENGER ACTIVITY IN RNA PREPARATIONS FROM CALF LENS CELLS AND RAT LIVER

6.1 INTRODUCTION

In this chapter it will be shown that it was possible for the amino acid incorporation to be stimulated by the addition of calf lens and rat liver RNA preparations to the cell-free system of *E. coli*. This stimulation was strongly dependent on the type of RNA preparations added. As no detailed characteristics regarding the messenger activity of these RNA preparations were known, these were determined first. In addition, some other special characteristics of these RNA preparations will be described.

The results obtained with RNA preparations isolated from calf lens and rat liver will be described separately and will be compared with each other in section 6.4 and in the discussion (chapter 8).

6.2 CHARACTERISTICS OF CALF LENS RNA

6.2.1 *Recoveries and relative DNA and protein concentrations of RNA preparations from calf lens cells*

In our preliminary experiments we started to isolate RNA from whole cortical and epithelial cells. As the lens is a tissue rich in protein, the RNA extraction procedure was performed in the presence of 1% SDS. However, these RNA preparations were not only heavily contaminated with DNA, but also had a high A 260/A 280 ratio (± 3.6) and the total amount of the A 260 absorbance units were unusually high (10,000 per 1000 lenses).

Dialysis of these RNA preparations against water, or gel filtration on a Sephadex G-50 column, revealed that most of the material absorbing at 260 mμ (± 95%) had a low molecular weight. After dialysis the A 260/A 280 ratio decreased to the normal value (A 260/A 280 = ± 2). Chromatography of this low molecular weight material on Dowex 1 × 2 columns (Schmitz, 1954) or electrophoresis on paper, revealed that most of the low molecular weight material was adenosine-5'-triphosphate (figure 16). An almost identical result was obtained when RNA was extracted (in the presence of 1% SDS) from the 10,000 g supernatant. This confirms that the low molecular weight material is not a degradation product of RNA.

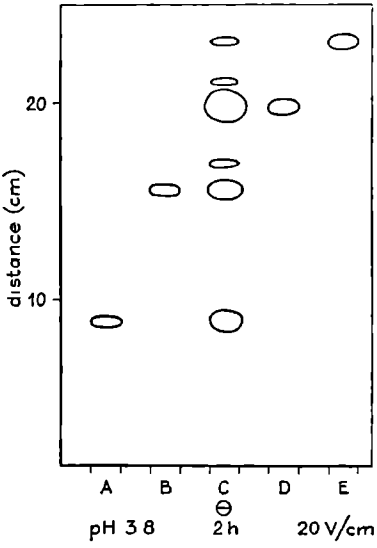

FIGURE 16 High voltage paper electrophoresis of lens nucleotides at pH 3 8 (0 05 M sodium citrate)

Electrophoresis was performed for 2 hours at 1000 volt Other conditions are given in 4 7 The samples applied were A Adenosine-5'-monophosphate, B Adenosine-5'-diphosphate, C Lens nucleotides, D Adenosine-5'-triphosphate, E Guanosine-5'-triphosphate

Similar observations were made by J Papaconstantinou (personal communication), who isolated RNA from chick lenses (see also Klethi and Mandel, 1965). These findings are probably related to the absence of enzyme systems capable of hydrolysing ATP (Green et al , 1955, van Heyningen, 1955) Bearing in mind these results and also that we wished to compare the messenger activities of our lens RNA preparations with those of RNA preparations isolated in the same way from rat liver, we decided to perform the separation of RNA from the crude nuclear pellet in the absence of SDS

It was reasonable to expect the RNA preparations isolated from the crude nuclear pellet to be contaminated with DNA To be sure that all DNA was removed, these RNA fractions were precipitated with 10% NaCl and also incubated with electrophoretically pure DNase in a concentration of 5µg per ml (25°C, 15 min) Extractions performed at a low temperature (30° – 40°C) and at pH 7 6 removed most RNA from cytoplasmic origin, whereas

RNA of nuclear origin could be extracted at a higher temperature (65°C at pH 8.3).

It was very difficult to prepare epithelial cells uncontaminated with the black pigment (melanin) of the iris. However, this pigment could be almost completely removed by gel filtration on a Sephadex G-25 column.

The RNA recoveries, and the DNA and protein contents of the different RNA preparations isolated from epithelial and cortical lens cells are summarized in table 9. From this table it can be concluded that the recovery of 65° RNA, after previous extractions at 39°C and 32°C (twice), is very low. For this reason it was decided to perform only one extraction at 39°C before the extraction at 65°C. Under these conditions the recoveries (table 9) were higher and as we needed a manageable quantity of RNA with which to perform subsequent experiments, it was decided to use this latter extraction procedure for the isolation of lens nuclear RNA, that is, RNA derived from the nuclei of lens cells – not RNA from the nuclear region of the lens as a whole. The recovery of RNA isolated from lens polysomes was nearly 100%. The values for DNA and protein contamination given in table 9 are the highest we found and in many cases the contamination was even lower.

TABLE 9. Recoveries and relative DNA and protein concentrations of RNA preparations isolated from calf lens cells.

RNA fraction	RNA	DNA	Protein	A 260 / A 280
	mg/1,000 lenses	%	%	
Polysomal RNA	2.76	0	< 1	1.93
32° RNA (pH 7.6)	0.5	< 1	< 3	2.0
39° RNA (pH 7.6)	15.0	< 2	< 3	1.97
65° RNA (pH 8.3) *	0.06	—	—	1.85
65° RNA (pH 8.3) **	0.5	< 2	< 3	1.86
0° RNA (pH 7.6) +	15.5	< 1	< 2	2.01
39° RNA (pH 8.3) +	0.6	< 1	< 2	1.89

DNA and protein concentrations are given as percentage of the combined RNA, DNA and protein.
The given temperature and pH indicates under which conditions the extraction of RNA was performed.

* Extraction at 65°C was performed after previous extractions at 39°C and 32°C (pH 7.6).
** Extraction at 32°C (pH 7.6) was omitted.
\+ Extracted according to the method of Hadjivassiliou and Brawerman (1965).

TABLE 10 Stimulation of amino acid incorporation by RNA preparations isolated from calf lens cells when added to the cell-free system of *E coli*

Addition (160 μg)	Amino acid incorporation counts/min/mg protein	Relative stimulation
None	630	—
None, deproteinized at zero time	122	—
Polysomal RNA	1240	2 0–fold
32° RNA (pH 7 6)	1870	3 0–fold
39° RNA (pH 7 6)	2000	3 2–fold
65° RNA (pH 8 3) *	—	> 8–fold
65° RNA (pH 8 3) **	5000	8 0–fold
0° RNA (pH 7 6) +	1940	3 1–fold
39° RNA (pH 8 3) +	2047	3 2–fold
tRNA (*E coli* B)	640	—

The reaction mixture contained the components as described in table 6 Incubations were performed in the presence of 8 nmoles DL-[l-^{14}C]-leucine (specific activity 55 2 mCi/mmole) and 3 5 μmoles magnesium acetate Samples were counted in a liquid scintillation spectrometer

* Extraction at 64°C was performed after previous extractions at 39°C and 32°C (pH 7 6)
** Extraction at 32°C (pH 7 6) was omitted
\+ Extracted according to the method of Hadjivassiliou and Brawerman (1965)

6 2 2 *Messenger activity of the RNA preparations from calf lens cells*

All isolated RNA preparations were tested for messenger activity The criterion used for the detection of messenger RNA was the capacity of the RNA fraction in question to stimulate the amino acid incorporation into protein in the cell-free system of *E coli*

The results obtained with RNA preparations isolated according to different procedures are presented in table 10 For comparison, results obtained with RNA preparations isolated according to the procedure of Hadjivassiliou and Brawerman (1965) (0° RNA, pH 7 6 and 39° RNA, pH 8 3) are also given It can be concluded that in all RNA preparations messenger activity is present, but that the extent of stimulation of amino acid incorporation varies with the isolation procedure The relative stimulation is strongly related to the amount of endogenous incorporation As the latter may vary an even higher relative stimulation of 2 to 3 times could sometimes be observed The highest messenger activity was always found

in the RNA fraction extracted from the crude nuclear pellet at 65°C and pH 8.3. These data show that "hot phenol" extraction at alkaline pH yields RNA with high messenger activity and that the nuclei contain a high content of messenger RNA. RNA preparations isolated at 39°C and pH 8.3 (see table 10) did not have as high a stimulatory activity as could be expected from the results obtained by Hadjivassiliou and Brawerman (1965) using analogously isolated rat liver RNA preparations. RNA fractions isolated from lens tissue culture cells (the generous gift of Prof. C. Jerusalem, Laboratory of Cyto-Histology, Nijmegen), had only a slight stimulatory effect on the amino acid incorporation. This observation is probably due to the very low amount of RNA which was isolated.

The properties of some of the above mentioned RNA preparations will be discussed in the next sections.

6.2.3 *Magnesium dependence of the amino acid incorporation directed by calf lens RNA*

For optimal assay conditions for messenger RNA one should determine the magnesium ion concentration at which the highest amino acid incorporation can be attained.

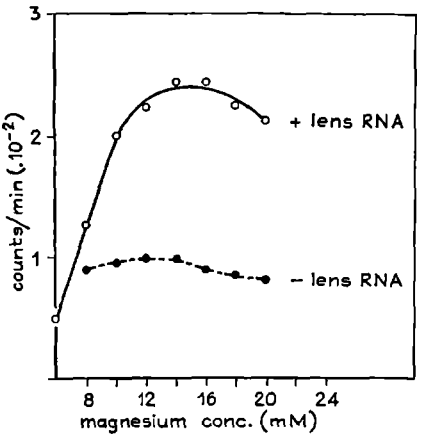

FIGURE 17. Effect of various magnesium concentrations on the amino acid incorporation in the presence and absence of calf lens polysomal RNA.

The reaction mixture contained the components as described in table 6. Incubations were performed in the presence of 6 nmoles DL-[1-^{14}C]-leucine (specific activity 34 mCi/mmole) and 2 nmoles unlabelled L-leucine. Samples were counted in a thin window gas flow counter.

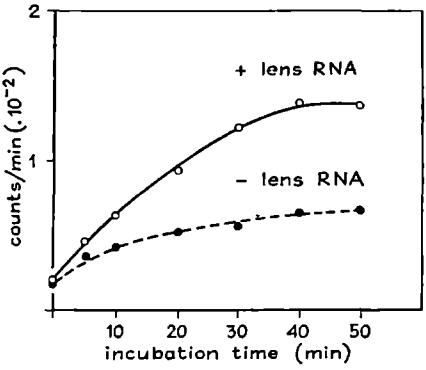

FIGURE 18. Kinetics of amino acid incorporation in the presence and absence of calf lens polysomal RNA.

The reaction mixture contained the components as described in table 6. Incubations were performed in the presence of 160 µg lens polysomal RNA, 6 nmoles DL-[l-^{14}C]-leucine (specific activity 34 mCi/mmole) and 2 nmoles unlabelled L-leucine. Samples were counted in a thin window gas flow counter.

From figure 17 it can be concluded that the optimal amino acid incorporation was reached at a magnesium concentration of 14–16 mM. This optimum does not deviate much from the magnesium optimum of the endogenous mRNA directed amino acid incorporation (figure 7).

6.2.4 *Kinetics of the lens RNA directed amino acid incorporation*

The rate of amino acid incorporation into protein was nearly constant during the first 20 minutes and declined soon after that time. Maximal incorporation was reached after about 40 minutes of incubation (figure 18). As it could not be excluded that the incorporation ceased as a result of breakdown of messenger RNA, we tried to find out whether the incorporation could be re-initiated by the addition of new RNA. The results shown in table 11 indicate that this was not the case. Stepwise addition of RNA to the incubation mixture rather diminished the final incorporation level. From these observations it can be concluded that re-initiation could not be achieved in this way. This result is probably related to the termination process of protein synthesis (see discussion in chapter 8). However, it might well be that these effects are partly the result of a decrease of the amino acid incorporation capacity of the cell-free system rather than breakdown of the messenger RNA itself. (see 6.3.5).

TABLE 11. Effect of stepwise addition of lens RNA to the incubation mixture on amino acid incorporation in the cell-free system of *E. coli*.

Addition	Incubation time (minutes)	Amino acid incorporation counts/min/mg protein	Relative stimulation
None	45	223	—
None, deproteinized at zero time	0	30	—
170 µg lens polysomal RNA added at zero time	45	527	2.4–fold
170 µg lens polysomal RNA added stepwise after 0', 15' and 30 min of incubation	45	442	2.0–fold
None	75	246	—
170 µg lens polysomal RNA added stepwise after 0', 15' and 30 min of incubation	75	493	2.0–fold
340 µg lens polysomal RNA added stepwise after 0' and 30 min of incubation	75	570	2.3–fold

The reaction mixture contained the components as described in table 6.
Incubations were performed in the presence of 10 nmoles DL-[l-^{14}C]-leucine (specific activity 34 mCi/mmole) and 5 nmoles unlabelled L-leucine.
Samples were counted in a thin window gas flow counter.

6.2.5 *Lens RNA concentration dependence of the amino acid incorporation*

From figure 19 it can be concluded that the amino acid incorporation was proportional to the amount of lens RNA added up to a concentration of 1 mg per ml. In a few cases saturation was not reached until somewhat higher RNA concentrations (figure 20).

6.2.6 *Effect of heating and rapid cooling on the messenger activity of calf lens RNA*

It is well known that RNA molecules can form complexes, or aggregates,

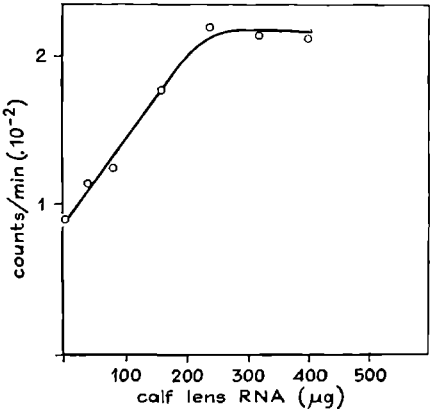

fig. 19

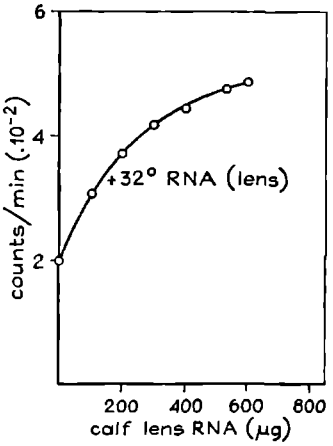

fig. 20

FIGURE 19. Effect of lens polysomal RNA concentration on amino acid incorporation in the cell-free system of *E. coli*.

The reaction mixture contained the components as described in table 6 except that the RNA concentration varied as indicated. Incubations were performed in the presence of 6 nmoles DL-[l-^{14}C]-leucine (specific activity 34 mCi/mmole) and 2 nmoles unlabelled L-leucine. Samples were counted in a thin window gas flow counter.

FIGURE 20. Effect of lens 32° RNA (pH 7.6) concentration on amino acid incorporation in the cell-free system of *E. coli*.

The reaction mixture contained the components as described in table 6 except that the RNA concentration varied as indicated.
Incubations were performed in the presence of 6 nmoles DL-[l-^{14}C]-leucine (specific activity 34 mCi/mmole) and 2 nmoles unlabelled L-leucine. Samples were counted in a thin window gas flow counter.

especially at high salt concentrations (Hayes et al., 1963; 1966a; 1966b; Wagner et al., 1967). Parish and Kirby (1966) have described experiments from which they concluded that complex formation between ribosomal and pulse labelled RNA took place. (See also Hastings et al., 1968; Kruh, 1967). As it could not be ruled out that messenger activity was masked due to complex formation between messenger RNA and ribosomal RNA (which is virtually always present in RNA preparations), we tried to reach a higher amino acid incorporation with the aid of heating and rapid cooling.

From the results given in table 12 it can be concluded that heating and rapid cooling had almost no effect on the messenger activity of these RNA preparations Only in the case of polysomal RNA is there a slight increase (20%) in messenger activity However, heating and rapid cooling affected other properties of these RNA preparations This will be described in the next chapter

TABLE 12 Effect of heating and rapid cooling on the messenger activity of RNA fractions isolated from calf lens

Addition (200 µg)	Amino acid incorporation counts/min/mg protein
None	633
None, deproteinized at zero time	143
Polysomal RNA	1166
Polysomal RNA (heated)	1564
39° RNA (pH 7 6)	2480
39° RNA (pH 7 6) (heated)	2445

Heating was performed in 10 mM Tris-HCl buffer, pH 7 8, containing 60 mM NH_4Cl for 15 minutes at 65°C After heating the RNA fractions were immediately cooled in ice water and tested for stimulatory activity The reaction mixture contained the components as described in table 6
Incubations were performed in the presence of 8 nmoles DL-[l-^{14}C]-leucine (specific activity 55 2 mCi/mmole) Samples were counted in a liquid scintillation spectrometer

6 2 7 *Ratios of the absolute amounts of different amino acids incorporated in the presence and absence of calf lens RNA*

The primary structure and consequently also the amino acid composition of a protein is determined by a specific messenger RNA molecule Messenger RNA molecules with different base sequences code for proteins with different primary structures and amino acid compositions As the cell-free system of *E coli* cannot be freed completely from endogenous messenger RNA, we investigated whether there was a difference in the proportion of incorporation of different amino acids in the presence and absence of lens RNA

From the results given in table 13 it can be concluded that there was a difference in the ratios of incorporation of different amino acids There was also a difference in the ratios of incorporations if different mixtures of two ^{14}C-amino acids were incubated separately In addition to the total

TABLE 13. Ratios of the incorporation of different amino acids into protein in the presence and absence of lens RNA.

RNA fraction	met	leu	asp	phe
Endogenous	78 (1)	200 (2.6)	78 (1)	44 (0.6)
39° RNA (pH 7.6)	93 (1)	815 (41)	106 (1.9)	195 (10)
	met	leu	asp	glu
Endogenous	20 (1)	279 (14)	2 (0.1)	19 (1)
Polysomal RNA	63 (1)	569 (6.7)	67 (1.5)	86 (1.5)
	met + leu	met + asp	asp + leu	leu + glu
Endogenous	476 (1)	16 (0.03)	442 (0.93)	332 (0.7)
Polysomal RNA	418 (1)	265 (0.6)	610 (1.5)	988 (2.4)

The reaction mixture contained the components as described in table 6.
Incubations were performed in the presence of 4 nmoles L-[Me-^{14}C]-methionine, 8 nmoles DL-[I-^{14}C]-leucine, 4 nmoles L-[^{14}C]-aspartic acid (uniformly labelled), 4 nmoles L-[^{14}C]-phenylalanine (uniformly labelled) and 8 nmoles DL-[I-^{14}C]-glutamic acid. The specific activity of all amino acids was 34 mCi/mmole.
The data are given as absolute amounts of measured counts.
Values between brackets indicate relative incorporations.

amounts of measured counts the relative incorporation values are also given. These results are an additional confirmation of the presence of messenger RNA in the different lens RNA preparations.

6.2.8 *Effect of lens RNA on the poly U directed phenylalanine incorporation*

The relative stimulation of the amino acid incorporation directed by lens polysomal RNA was usually not very high (2 to 3 times). This results from the fact that the major part of lens polysomal RNA is from ribosomal origin and it is generally assumed that mature ribosomal RNA, in contrast to nascent rRNA (see Crick, 1963; Gros et al., 1963; Otaka et al., 1964; Nakada, 1965; Muto, 1968) does not function as template. It cannot be excluded, however, that ribosomal RNA blocks the amino acid incorporation by non-specific binding to the messenger RNA binding site of the ribosome. This inhibitory effect of ribosomal RNA can also be expected on the poly U directed phenylalanine incorporation. Extra addition of lens RNA to the incubation mixture, however, had no inhibitory effect on the phenylalanine incorporation. On the contrary, a strong stimulatory effect was observed. These results were not dependent on the RNA fraction studied, or on the sequence of addition of RNA and poly U to the incubation mixture (table 14).

TABLE 14 The stimulatory effect of combined addition of poly U and lens RNA on phenylalanine incorporation

Addition	Amino acid incorporation counts/min/mg protein	Relative stimulation
None	15	—
Poly U (30 μg)	4489	299–fold
Lens 39° RNA (pH 7 6, 200 μg)	78	5 2–fold
Lens 39° RNA (pH 7 6, 200 μg) and Poly U (30 μg)	6753	450–fold
Lens polysomal RNA (200 μg)	58	3 9–fold
Lens polysomal RNA (200 μg) and Poly U (30 μg)	7134	496–fold

The reaction mixture contained the components as described in table 6
Incubations were performed in the presence of 4 5 μmoles magnesium acetate, 0 087 nmoles L-^{14}C-phenylalanine (uniformly labelled, 504 mCi/mmole) and 10 nmoles unlabelled L-phenylalanine
Samples were counted in a thin window gas flow counter

TABLE 15 The stimulatory effect of combined addition of poly U and lens RNA on valine and alanine incorporation

Addition	Amino acid incorporation counts/min/mg protein	Relative stimulation
None	70	—
Lens 39° RNA (pH 7 6, 200 μg)	322	4 6–fold
Lens 39° RNA (pH 7 6, 200 μg) and poly U (30 μg)	332	4 7–fold
Lens polysomal RNA (200 μg)	195	2 8–fold
Lens polysomal RNA (200 μg) and poly U (30 μg)	205	2 9–fold
Poly U (30 μg)	77	—

The reaction mixture contained the components as described in table 6
Incubations were performed in the presence of 3 5 μmoles magnesium acetate, 20 nmoles DL-[^{14}C]-valine (uniformly labelled, 10 mCi/mmole) and 20 nmoles DL-[^{14}C]-alanine (uniformly labelled, 10 mCi/mmole)
Samples were counted in a liquid scintillation spectrometer

TABLE 16 Effect of rat liver RNase inhibitor on the poly U directed phenylalanine incorporation

Addition	Amino acid incorporation counts/min/mg protein	Relative stimulation
None	286	—
None, deproteinized at zero time	193	—
Poly U (60 µg)	47,060	164–fold
Poly U (60 µg) and RNase inhibitor (100 µg)	41,150	144–fold
RNase inhibitor (100 µg)	325	—

The reaction mixture contained the components as described in table 6
Incubations were performed in the presence of 4 5 µmoles magnesium-acetate, 10 nmoles unlabelled L-phenylalanine and 0 087 nmoles L-^{14}C-phenylalanine (uniformly labelled, 504 mCi/mmole)
Samples were counted in a liquid scintillation spectrometer
Partly purified RNase inhibitor was a generous gift of Drs Yessie Gribnau (Laboratorium voor Biochemie, Nijmegen)

Addition of poly U to the lens RNA directed valine and alanine incorporation, had virtually no influence on the amount of incorporation (table 15), although sometimes a slight inhibitory effect could be observed. Interpretation of these results in terms of interference with endogenous RNase would be in contradiction to results obtained with purified RNase inhibitor isolated from rat liver (Gribnau et al, 1969) Addition of this inhibitor to the incubation mixture had no stimulatory effect on the amino acid incorporation (table 16) These effects will be discussed further in chapter 8

6 2 9 *Effect of lens RNA on the amino acid incorporation in a cell-free system prepared from rat liver*

As our RNA preparations were from mammalian origin, it seemed reasonable to assume that addition of these preparations to a cell-free system prepared from a mammalian tissue would stimulate the amino acid incorporation From the results presented in table 17 it can be concluded that such an addition had no stimulatory effect on the amino acid incorporation, although addition of RNA to a non preincubated system had a slight stimulatory effect Very similar results were obtained when these RNA

preparations were added to a cell-free system prepared from the calf lens according to Bloemendal et al (1968)

As it could not be excluded that the 105,000 g supernatant prepared

TABLE 17 Effect of lens RNA on amino acid incorporation in a cell-free system prepared from rat liver

Addition	Amino acid incorporation counts/minute		Relative stimulation
	Preincubated	Non-preincubated	
None	232	1108	5 6–fold
None, deproteinized at zero time	209	209	—
Minus supernatant	63	108	—
Lens polysomal RNA (150 µg)	266	1485	7 0–fold
Lens 39° RNA (pH 7 6, 150 µg)	230	1719	8 2–fold
Liver tRNA (50 µg)	210	1272	6 0–fold

The reaction mixture contained the components as described by Bloemendal et al (1966) Incubations were performed in the presence of 100 µg polysomes, 100 µl of 105,000 g supernatant, 2 nmoles magnesium acetate, 4 nmoles DL-[l-^{14}C]-leucine (specific activity 34 mCi/mmole) and 8 nmoles unlabelled L-leucine Preincubations were carried out for 30 min at 37°C in the absence of additional RNA and ^{14}C-amino acids

Incubations were performed for 30 minutes at 37°C Samples were counted in a liquid scintillation spectrometer

TABLE 18 Effect of the 105,000 g supernatant of calf lens cells on the lens RNA directed amino acid incorporation in the cell-free system of *E coli*

Addition	Amino acid incorporation counts/minute	Δ cpm
None	276	—
Polysomal RNA (170 µg)	550	274
Supernatant	558	—
Supernatant and polysomal RNA (170 µg)	844	254

The reaction mixture contained the components as described in table 6

Incubations were performed in the presence of 4 nmoles unlabelled L-methionine, 2 nmoles L-[Me-^{14}C]-methionine (specific activity 110 mCi/mmole) and where indicated 50 µl of 105,000g supernatant (5 mg protein) Samples were counted in a liquid scintillation spectrometer

from the calf lens contained factors such as a specific initiator – tRNA, necessary for the efficient functioning of messenger RNA from animal sources when added to a bacterial cell-free system, we studied the effect of addition of lens supernatant to the cell-free system of *E coli* on the lens RNA directed amino acid incorporation However, the results presented in table 18 show that lens supernatant had no stimulatory effect on the amino acid incorporation A possible explanation of these results will be given in chapter 8

6 2 10 *Effect of ribonucleoprotein particles (RNP-particles) on the amino acid incorporation in the cell-free system of E coli*

There is indirect evidence that messenger RNA is transported from the nucleus to the cytoplasm as a ribonucleoprotein particle (Henshaw et al, 1965, Spirin et al, 1965, Georgiev, 1967, Perry and Kelley, 1966, 1968, Penman et al, 1968) It is thought that this particle is bound as such to the ribosome without prior removal of the protein Several investigators have tried to isolate this ribonucleoprotein particle by treating polysomes with EDTA (Huez et al, 1967, Henshaw, 1968, Temmerman and Lebleu, 1969). Weisberger et al (1966) reported results from which they concluded that such particles isolated from reticulocyte polysomes were active in stimulating

TABLE 19 Effect of RNP-particles prepared from calf lens and rat liver polysomes on amino acid incorporation in the cell-free system of *E coli*

Addition	Amino acid incorporation counts/minute	Relative stimulation
None	230	—
RNP lens (30 µg)	450	2–fold
RNP liver (50 µg)	430	1 9–fold
RNP lens (30 µg) minus ribosomes	270	—
RNP liver (50 µg) minus ribosomes	241	—

The reaction mixture contained the components as described in table 6
Incubations were performed in the presence of 2 nmoles of unlabelled L-glutamic acid, L-arginine and L-glycine, 20 nmoles DL-^{14}C-glutamic acid (specific activity 15 2 mCi/mmole), 10 nmoles L-^{14}C glycine (specific activity 8 1 mCi/mmole) and 10 nmoles L-^{14}C-arginine (specific activity 19 5 mCi/mmole)
Samples were incubated for 60 minutes at 37 °C and counted in a thin window gas flow counter

the amino acid incorporation into protein in the homologous cell-free system They were also able to show that the protein synthesized was haemoglobin (see also Armentrout et al , 1966, 1968)

We attempted to repeat these experiments with RNP-particles isolated from lens and liver polysomes The messenger activity was again tested in the cell-free system prepared from *E coli* From the results presented in table 19 it can be concluded that these particles stimulated the amino acid incorporation This stimulation, however, was sometimes nearly equal to the incorporation in a control experiment performed in the absence of *E coli* ribosomes The maximal incorporation in this case was also reached at a magnesium concentration of 14 mM The amount of incorporation, however, was not much higher than that obtained with lens polysomal RNA In addition, the stimulation of amino acid incorporation was not always reproducible

6 2 11 *Fractionation of lens RNA preparations with messenger activity*

The RNA preparations appeared to be a mixture of RNA molecules with different characteristics In the literature several fractionation procedures are described (Oberg et al , 1965, Lichtenstein et al , 1967, Young, 1968, Sedat et al , 1967), although not all of them are suitable for use in the present study (Kirby, 1960, Hastings et al , 1965) The method we decided to use was sucrose density gradient centrifugation

Density gradient centrifugation of lens polysomal RNA revealed two major peaks with sedimentation values of 18S and 28S (figure 21) The sedimentation pattern was similar to those described in the literature for polysomal RNA preparations from other tissues Only a small amount of material absorbing at 260 mμ was present in the region near to the top of the gradient, which indicated that material of low molecular weight such as tRNA and oligonucleotides, was virtually absent Assays of messenger activity of the RNA fractions from the sucrose gradient of lens polysomal RNA, revealed that there was a slight increase of specific activity in the 10S region but that the highest specific activity was concentrated in fractions near to and at the bottom of the gradient (figure 21)

Analysis of lens RNA extracted at 39 °C (pH 7 6) revealed a peak absorbing at 260 mμ in the 18S region which was usually higher than the peak in the 28S region (Figure 22) Assays of messenger activity of the corresponding RNA fractions revealed a slight increase in specific activity in the 10S region but the highest specific activity was found in fractions near to and at the bottom of the gradient (figure 22)

Sucrose density gradient analysis of lens RNA extracted at 65 °C (pH 8 3)

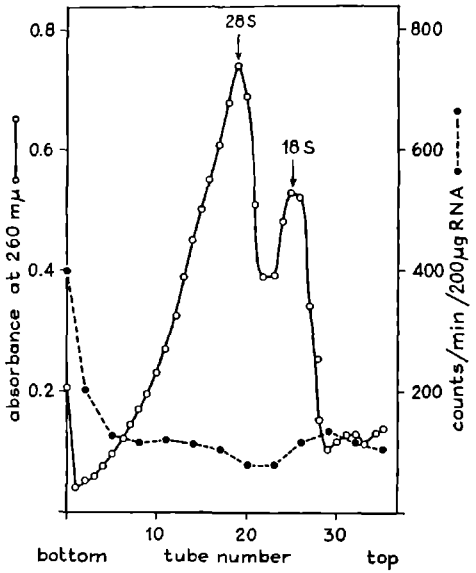

FIGURE 21. Sedimentation pattern of RNA from lens polysomes and ability of RNA fractions to stimulate amino acid incorporation into protein.

The reaction mixture contained the components as described in table 6. Incubations were performed in the presence of 8 nmoles DL-[l-^{14}C]-leucine (specific activity 34 mCi/mmole) and 2 nmoles unlabelled L-leucine. The samples were counted in a thin window gas flow counter.
Incorporated radioactivity in the absence of added RNA has been subtracted from all experimental values.
o———o: absorbance at 260 mμ.
●- - - -●: specific activity (counts/min/200 μg RNA).

revealed a sedimentation pattern which was strongly different from the patterns mentioned above. RNA was found over the whole gradient and with a maximum 260 mμ absorbance in the 18S region. This maximum was preceded by a small shoulder and followed by a broad one (figure 23). From analysis of messenger activity of the corresponding RNA fractions from the sucrose gradient, it can be concluded that there was a slight increase of messenger activity in the 20S region, but yet again the highest messenger activity was found in fractions towards the bottom of the tube (figure 23). The messenger activity of a few fractions of the gradient could not be determined due to the low RNA content.

The regions of highest messenger activity from the sucrose gradients of

the different RNA preparations are very similar. This will be discussed in more detail in chapter 8.

Another technique frequently used to separate RNA molecules is chromotgraphy on methylated-albumin kieselguhr columns (MAK-column) according to Mandell and Hershey (1960). Using this column a good separation can be achieved between DNA, tRNA and the two ribosomal RNA's from bacterial origin. (Ishihama et al., 1962; Sueoka and Cheng, 1962). A disadvantage with this method is that in most cases a large amount of RNA sticks to the column and is difficult to elute without degradation. In addition, as a result of the high salt concentration used for elution, complexes of RNA are formed (Ellem, 1966; Ingle and Key, 1968).

In spite of these drawbacks we tried to fractionate lens polysomal RNA on these columns. From figure 24 it can be concluded that the result was

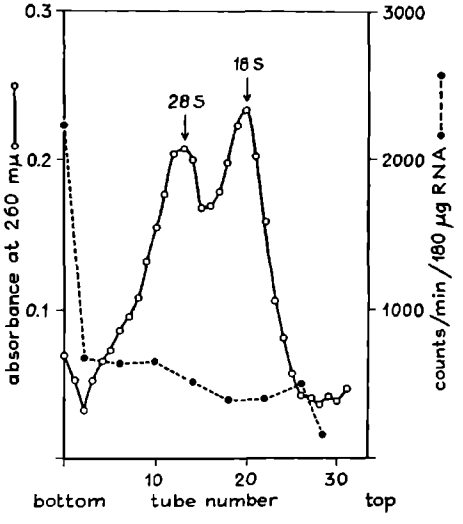

FIGURE 22. Sedimentation pattern of lens 39° RNA (pH 7.6) and ability of RNA fractions to stimulate amino acid incorporation into protein.

The reaction mixture contained the components as described in table 6. Incubations were performed in the presence of 10 nmoles DL-[l-^{14}C]-leucine (55.2 mCi/mmole). The samples were counted in a thin window gas flow counter.
Incorporated radioactivity in the absence of added RNA has been subtracted from all experimental values.
o———o: absorbance at 260 mμ.
●– – – –●: specific activity (counts/min/180 μg RNA).

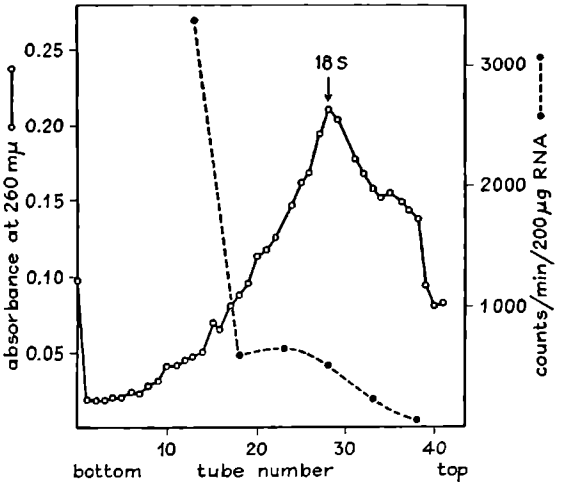

FIGURE 23. Sedimentation pattern of RNA from calf lens (extracted at 65°C pH 8.3) and ability of RNA fractions to stimulate amino acid incorporation.

The reaction mixture contained the components as described in table 6. Incorporations were performed in the presence of 20 nmoles DL-[l-^{14}C] leucine (specific activity 55.2 mCi/mmole). Samples were counted in a liquid scintillation spectrometer.
Incorporated radioactivity in the absence of added RNA has been subtracted from all experimental values.
o———o, absorbance at 260 mμ.
●– – – –●, specific activity (counts/min/200 μg RNA).

not very satisfactory (cf. Urban et al., 1965). Nearly all the material absorbing at 254 mμ was concentrated in one peak. We examined different fractions from this peak for messenger activity and the results in table 20 indicate a virtually homogeneous distribution of activity throughout the peak.

6.2.12 *Effect of lens RNA on the binding of N-acetylmethionyl-tRNA and methionyl-tRNA to E. coli ribosomes*

Most lens proteins are blocked in the N-terminal position (Hoenders et al., 1967; 1968a; Mok and Waley, 1968). One of these proteins, α-crystallin, which represents about 30% of the total, has acetyl-Met-Asp-Ile-Ala-as the N-terminal sequence (Hoenders et al., 1968b). Attempts were made to detect stimulation of ^{14}C-acetate incorporation into protein in the *E. coli*

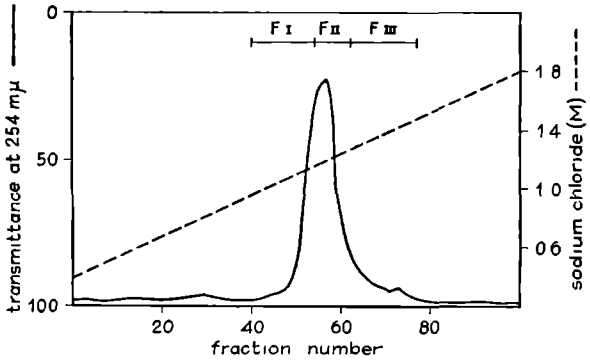

FIGURE 24 Chromatography of lens polysomal RNA on methylated albumin kieselguhr column
Lens polysomal RNA (3mg) was applied to a MAK column (1 5 × 10 cm) equilibrated with 0 05 M phosphate buffer, pH 7 6, containing 0 4 M NaCl A linear gradient was applied ranging to 1 8 M NaCl in the same buffer, mixing reservoir 300 ml Elution was accomplished at room temperature at 1 5 ml/h The effluent was monitored at 254 mμ using an LKB Uvicord absorptiometer The fraction volume was 6 ml Fractions were collected as indicated and RNA present was precipitated with 2 5 volumes cold ethanol (96%)

TABLE 20 Messenger activity of RNA fractions obtained after chromotography of lens polysomal RNA on a MAK-column

Addition	Amino acid incorporation counts/min/mg protein	Relative stimulation
None	190	—
None, deproteinized at zero time	67	—
Lens polysomal RNA (150 μg)	441	2 3–fold
Fraction FI (170 μg)	343	1 7–fold
Fraction FII (170 μg)	275	1 4–fold
Fraction FIII (170 μg)	271	1 4–fold

The reaction mixture contained the components as described in chapter 6
Incubations were performed in the presence of 3 5 μmoles magnesium acetate, 10 nmoles DL-[1-^{14}C]-leucine (specific activity 34 mCi/mmole) and 5 nmoles unlabelled L-leucine
Samples were counted in a thin window gas flow counter FI, FII and FIII are the pooled fractions obtained after MAK-column chromatography (figure 25)

TABLE 21 Investigations on the incorporation of N-acetylmethionine and methionine from N-acetylmethionyl-tRNA and methionyl tRNA respectively, in the cell-free system prepared from calf lens cells

Addition	Amino acid incorporation counts/minute	
	N-acetyl-met-tRNA	met-tRNA
None	49	213
None, deproteinized at zero time	48	31
Puromycin (0 5 mM)	37	40
Minus polysomes	41	45
Minus GTP, ATP, PK and PEP	47	60
Minus ATP, PK and PEP	53	147
Minus amino acids, ATP, PK and PEP	73	177

The reaction mixture (0 5 ml) contained the components as described by Bloemendal et al (1968) Incubations were performed in the presence of 7 µmoles magnesium acetate, 180 µg lens polysomes, 1 5 mg lens supernatant protein, 150 µg N-acetyl-^{14}C-met-tRNA (0 45 nmoles ^{14}C-methionine/mg tRNA, specific activity 56 8 mCi/mmole) or 150 µg ^{14}C-met-tRNA (0 45 nmoles ^{14}C-methionine/mg tRNA, specific activity 56 8 mCi/mmole) tRNA was prepared from rat liver Synthesis of methionyl-tRNA was performed according to Konings et al (1968) Acetylation of methionyl-tRNA was performed according to the method of De Groot et al (1967) The amount of acetylation was over 95% (determined according to Schofield and Zamecnik, 1968, and Konings et al 1968) The reaction mixture was incubated for 45 minutes at 37° C Samples were counted in a liquid scintillation spectrometer

system after lens 105,000g supernatant and lens polysomal RNA had been added These attempts were not successful

It seemed reasonable to suppose that the *in vivo* synthesis of α-crystallin and possibly other lens proteins, starts with N-acetylmethionine (compare with N-formylmethionine in *E coli*) To test this idea we studied the incorporation of N-acetylmethionine from N-acetylmethionyl-tRNA into protein in the cell-free system prepared from calf lens cells Unfortunately the results given in table 21 do not allow any definite conclusions to be made Also included are the results obtained with methionyl-tRNA which confirm the usual findings of amino acid incorporation *in vitro*. More definite results were obtained in experiments to test the binding ,according to Nirenberg and Leder (1964), of N-acetylmethionyl-tRNA and methionyl-tRNA to ribosomes of *E coli* (figure 25) Addition of lens RNA had a stimulatory effect on the binding of both tRNA preparations

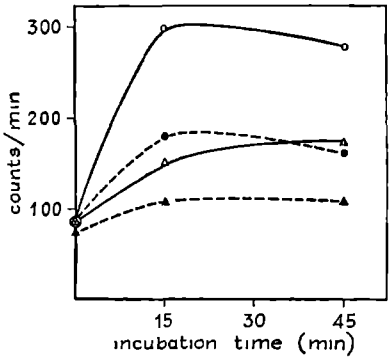

FIGURE 25 Effect of lens polysomal RNA on binding of methionyl-tRNA or N-acetylmethionyl-tRNA to *E coli* ribosomes

Incubations were performed in the presence of 200 µg *E coli* ribosomes, 200 µg lens polysomal RNA and 150 µg of ^{14}C-met-tRNA (0 45 µmole L-^{14}C-methionine/mg tRNA, specific activity 56 8 mCi/mmole) or N-acetyl-^{14}C-met-tRNA (0 45 µmole N-acetyl-^{14}C-methionine/mg tRNA, specific activity 56 8 mCi/mmole) The reaction mixtures were incubated in 10 mM Tris-HCl buffer, pH 7 8, containing 60 mM MH_4Cl and 14 mM magnesium acetate at 30° C and aliquots were removed at the times indicated Radioactivity measurements were performed according to Nirenberg and Leder (1964)

▲- - - -▲ reaction mixture supplemented with N-acetyl-^{14}C-methionyl-tRNA, no lens polysomal RNA added

●- - - -● reaction mixture supplemented with N-acetyl-^{14}C-methionyl-tRNA and lens polysomal RNA

△———△; reaction mixture supplemented with ^{14}C-methionyl-tRNA, no lens polysomal RNA added

o———o reaction mixture supplemented with ^{14}C-methionyl-tRNA and lens polysomal RNA

6 3 1 *Introduction*

The results described in the previous sections using calf lens RNA have been compared with the results obtained with rat liver RNA preparations We have also performed experiments with RNA preparations isolated from regenerating rat liver

After partial hepatectomy several biochemical changes can be observed The synthesis of RNA in regenerating rat liver is considerably enhanced as compared with normal "resting" liver (Welling et al , 1965) This increase occurs before the onset of DNA synthesis, which starts at about 18 hours after hepatectomy (Eliasson et al , 1951 , Hecht et al , 1956), reaches a maximum after about 24 hours (Nygaard et al , 1955 , Hecht et al , 1956), and is followed by intensive mitotic activity some hours later (Cater et al , 1956) The incorporation rate of labelled RNA precursors reaches a maximum within 20 hours after the operation, when DNA synthesis is still at a lower level (Anderson et al , 1956 , Holbrook et al , 1962 , Welling and Cohen, 1960)

One could expect RNA isolated at a certain time after partial hepatectomy to have a higher content of messenger RNA and consequently a higher specific activity than preparations from normal liver We also looked for

TABLE 22 Recoveries and relative DNA and protein concentrations of RNA preparations isolated from rat liver

RNA fraction	RNA mg/g liver	DNA %	Protein %	A 260 / A 280
Polysomal RNA	0 3	0	< 1	2 0
Microsomal RNA	1 0	< 2	< 3	2 12
32° RNA (pH 7 6)	5 6	< 2	< 2	2 0
39° RNA (pH 7 6)	4 1	0	< 1	1 96
65° RNA (pH 8 3)	0 5	< 2	< 3	1 87
39° RNA (pH 8 3) *	0 35	< 1	< 2	1 89
0° RNA (pH 7 6) **	0 031	< 2	< 1	1 90
39° RNA (pH 7 6) **	0 018	< 1	< 1	1 90
65° RNA (pH 8 3) **	—	> 95	—	—

DNA and protein concentrations are given as percentage of the combined RNA, DNA and protein

The given temperature and pH indicate under which conditions the extraction of RNA was performed

* Extracted according to the method of Hadjivassiliou and Brawerman (1965)

** Extracted from purified nuclei

any difference in messenger activity between RNA isolated from whole rat liver (65°C, pH 8.3) and RNA prepared from purified nuclei. The nuclei were prepared according to the method of Di Girolamo et al. (1964) and the extraction of RNA was performed with phenol at successively, 0°C (pH 7.6) at 39°C (pH 7.6) and at 65°C (pH 8.3).

6.3.2 *Recoveries and relative DNA and protein concentrations of RNA fractions from rat liver*

The amounts of RNA obtained from rat liver after extractions with phenol are given in table 22. The RNA preparations isolated from whole rat liver (65°C at pH 8.3) and from nuclei, were treated with DNase before a second phenol extraction was performed. The relative DNA and protein concentrations present in these RNA preparations are also shown.

The recoveries of RNA obtained from regenerating rat liver were lower than those obtained from an equivalent amount of normal rat liver. These results may be explained by the fact that regenerating rat liver partly consists of necrotic tissue which gives little or no contribution to the amount of RNA.

6.3.3 *Messenger activity of the RNA preparations from rat liver*

The messenger activities observed in the different RNA preparations isolated from rat liver are given in table 23. For comparison our results obtained with RNA preparations isolated according to Hadjivassiliou and Brawerman (1965), are included. For the same purpose, the messenger activities of RNA preparations from viral origin are also given.

Generally the extent of stimulation obtained in separate experiments varied. For this reason, liver 65° RNA (pH 8.3) was used as a standard in this study and was included in each series of messenger activity assays. With the use of this standard the activity values obtained in different experiments could be compared.

It can be concluded that:

a. The highest messenger activities were observed in RNA preparations isolated from the whole liver at high temperature (65°C, pH 8.3), and from purified nuclei;

b. the stimulatory activity of liver RNA preparations was of the same order of magnitude as similarly isolated RNA from calf lens (table 10);

c. using the same method of isolation, RNA preparations from regenerating rat liver have a lower messenger activity than those isolated from normal rat liver;

d. the amino acid incorporation is completely inhibited by RNase;
e. the amino acid incorporation was very strongly stimulated by the addition of RNA fractions of viral origin, indicating that the results obtained with our cell-free system are consistent with those obtained by other investigators.

In the next sections some characteristics of these isolated rat liver RNA preparations will be described.

TABLE 23. Stimulation of amino acid incorporation by RNA preparations isolated from rat liver when added to the cell-free system of *E. coli*.

Addition (160 µg)	Amino acid incorporation counts/min/mg protein	Relative stimulation
None	630	—
None, deproteinized at zero time	122	—
Polysomal RNA	1470	2.3–fold
Microsomal RNA	1254	2.0–fold
32° RNA (pH 7.6)	1590	2.5–fold
39° RNA (pH 7.6)	2510	4.0–fold
65° RNA (pH 8.3)	5400	8.6–fold
32° RNA (pH 7.6, 20 hr) *	1033	1.8–fold
39° RNA (pH 7.6, 20 hr) *	1363	2.2–fold
65° RNA (pH 8.3, 16 hr) *	2577	4.1–fold
65° RNA (pH 8.3, 20 hr) *	4115	6.5–fold
65° RNA (pH 8.3, 24 hr) *	4661	7.4–fold
39° RNA (pH 8.3) **	4154	6.6–fold
0° RNA (pH 7.6) +	5003	8.0–fold
39° RNA (pH 7.6) +	6858	10.8–fold
tRNA (liver)	570	—
tRNA (*E. coli* B)	640	—
TMV RNA (35 µg)	7596	12.1–fold
TYMV RNA (40 µg)	8321	13.2–fold
Rauscher virus RNA (? µg)	3617	5.7–fold
RNase (1 µg)	133	—

The reaction mixture contained the components as described in table 6.
Incubations were performed in the presence of 8 nmoles DL-[I-^{14}C]-leucine (specific activity 55.2 mCi/mmole). Samples were counted in a liquid scintillation spectrometer.

* RNA fractions obtained after partial hepatectomy according to Higgens and Anderson (1931).

** Extracted according to the method of Hadjivassiliou and Brawerman (1965).

\+ Extracted from purified nuclei.

6 3 4 *Magnesium dependence of the amino acid incorporation directed by rat liver RNA*

The amino acid incorporation directed by rat liver RNA was optimal at a magnesium concentration of 14 mM (figure 26). This optimum was quite similar to that for lens RNA (compare figure 17) Furthermore, this optimum did not deviate much from the magnesium optimum of the endogenous amino acid incorporation (figure 7)

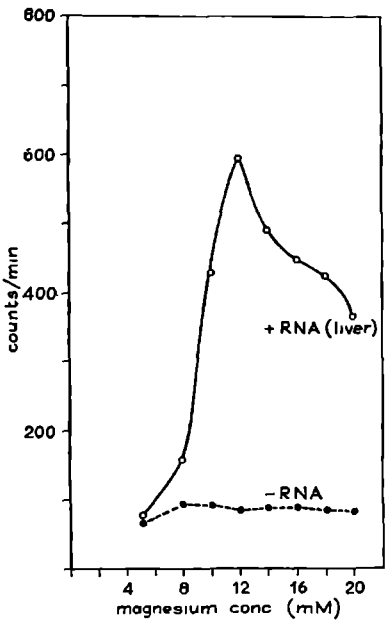

FIGURE 26 Effect of various magnesium concentrations on amino acid incorporation in the presence and absence of rat liver 65° RNA (pH 8 3)

The reaction mixture contained the components as described in table 6 except that the magnesium concentration varied as indicated Incubations were performed in the presence of 6 nmoles DL-[l-^{14}C] leucine (specific activity 34 mCi/mmole) and 2 nmoles unlabelled L-leucine
Samples were counted in a thin window gas flow counter

6 3 5 *Kinetics of amino acid incorporation directed by rat liver RNA*

The amino acid incorporation directed by liver RNA extracted at 65°C (pH 8 3) was linear up to 20 minutes and was almost complete after 45

minutes of incubation (figure 27). Addition of extra RNA after 45 minutes, or stepwise addition of RNA to the incubation mixture, had virtually no effect on the level of amino acid incorporation (table 24).

From these results it can be concluded that the capacity of the cell-free system to incorporate amino acid into protein decreased during the course of incubation.

One of the reasons may be denaturation or breakdown of molecules indispensable for protein synthesis.

Addition of an RNase inhibitor Gribnau et al. (1969) usually had no effect on the level of amino acid incorporation, although occasionally a slight inhibitory effect was even observed.

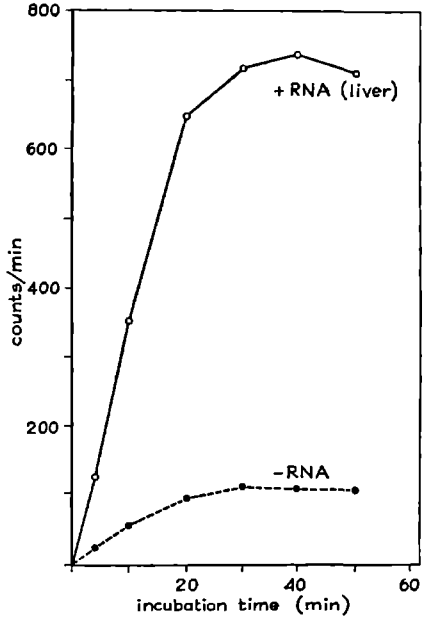

FIGURE 27. Kinetics of amino acid incorporation in the presence and absence of rat liver 65° RNA (pH 8.3). The reaction mixture contained the components as described in table 6. Incubations were performed in the presence of 6 nmoles DL-[l-^{14}C] leucine (specific activity 34 mCi/mmole) and 2 nmoles unlabelled L-leucine.
Samples were counted in a thin window gas flow counter.

TABLE 24. Effect of stepwise addition of liver RNA to the incubation mixture on amino acid incorporation in the cell-free system of *E. coli*.

Addition	Incubation time (minutes)	Amino acid incorporation counts/min/mg protein	Relative stimulation
None	45	208	—
None, deproteinized at zero time	0	30	—
200 µg 65° RNA (pH 8.3) added at zero time	45	2300	11.6–fold
200 µg 65° RNA (pH 8.3) added stepwise after 0', 15 and 30 min of incubation.	45	1807	8.7–fold
200 µg 65° RNA (pH 8.3) added after 15 min of incubation.	60	1340	6.4–fold
None	75	240	—
200 µg 65° RNA (pH 8.3) added after 30 min of incubation.	75	845	4.1–fold
400 µg 65° RNA (pH 8.3) added stepwise after 0' and 30 min of incubation.	75	2533	12.2–fold
400 µg 65° RNA (pH 8.3) added stepwise after 0' and 45 min of incubation.	90	2443	11.7–fold

The reaction mixture contained the components as described in table 6, except that the RNA concentration varied as indicated. Incubations were performed in the presence of 6 nmoles DL-[l-^{14}C]-leucine (specific activity 34 mCi/mmole) and 2 nmoles unlabelled L-leucine.
Samples were counted in a liquid scintillation spectrometer.

6.3.6 *RNA concentration dependence of the amino acid incorporation*

Addition of different amounts of rat liver RNA to the incubation mixture had a strong effect on the level of amino acid incorporation (figure 28). Depending on the RNA preparation studied dependency was found. For rat liver RNA extracted at 65°C (pH 8.3) an optimal incorporation was reached at a concentration of about 600 µg RNA per ml (figure 28), while for other RNA preparations (see figure 29) the optimum had not been reached even at a concentration of 1.2 mg per ml.

These differences are probably related to the specific messenger activity of the RNA fractions in question (compare with table 23).

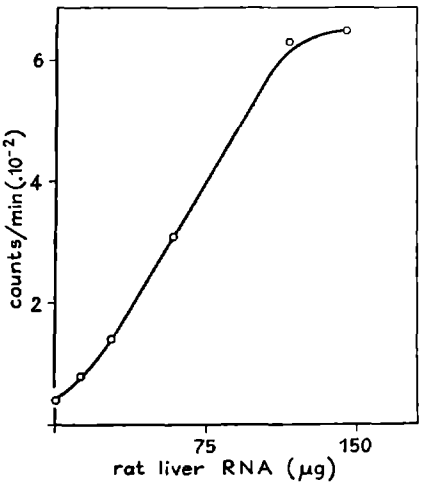

fig. 28

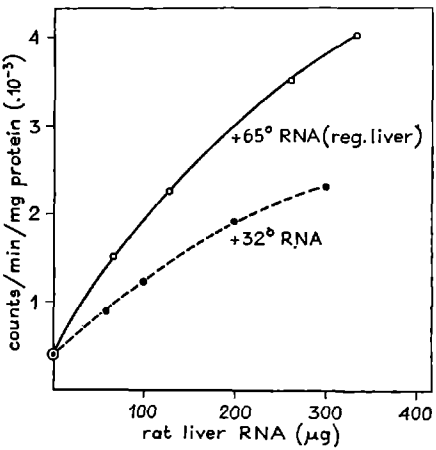

fig. 29

FIGURE 28. Effect of liver 65° RNA concentration on amino acid incorporation in the cell-free system of *E. coli*.
The reaction mixture contained the components as described in table 6 except that the RNA concentrations varied as indicated. Incubations were performed in the presence of 6 nmoles DL-[l-^{14}C] leucine (specific activity 34 mCi/mmole) and 2 nmoles unlabelled L-leucine.
Samples were counted in a thin window gas flow counter.

FIGURE 29. Effect of rat liver RNA concentration on amino acid incorporation in the cell-free system of *E. coli*.
The reaction mixture contained the components as described in table 6 except that the RNA concentration varied as indicated. Incubations were performed in the presence of 6 nmoles DL-[l-^{14}C]-leucine (specific activity 34 mCi/mmole) and 2 nmoles unlabelled L-leucine. Samples were counted in a liquid scintillation spectrometer.
o———o: reaction mixture supplemented with 65° RNA (pH 8.3) prepared from regenerating rat liver (24 hours after partial hepatectomy).
●– – – –●: reaction mixture supplemented with 32° RNA (pH 7.6).

6.3.7 *The effect of heating and rapid cooling on the messenger activity of rat liver RNA*

Heating at 65° C for 15 minutes and rapid cooling of liver RNA preparations had virtually no stimulatory effect on the amino acid incorporation in the

TABLE 25. Effect of heating and rapid cooling on the messenger activity of RNA preparations from rat liver.

Addition	Amino acid incorporation counts/min/mg protein	Relative stimulation
None	633	—
None, deproteinized at zero time	143	—
65° RNA (pH 8 3; 160 µg)	5782	9.1–fold
65° RNA (pH 8.3; 160 µg), (heated)	5490	8.7–fold
39° RNA (pH 7.6; 150 µg)	2480	4 0–fold
39° RNA (pH 7 6; 150 µg), (heated)	2444	3 9–fold

Heating was carried out in 10 mM Tris-HCl buffer, pH 7 8 containing 60 mM NH_4Cl for 15 minutes at 65°C. After heating the RNA fractions were immediately cooled in ice water and tested for stimulatory activity.
The reaction mixture contained the components as described in table 6.
Incubations were performed in the presence of 8 nmoles DL-[l-^{14}C]-leucine (specific activity 55.2 mCi/mmole).
Samples were counted in a liquid scintillation spectrometer.

cell-free system of *E. coli* (table 25). On the contrary, a slight inhibitory effect could be observed.

These results indicate that no messenger activity was masked due to intra- or inter-molecular base pairing.

6.3.8 *Ratios of the absolute amounts of different amino acids incorporated in the presence and absence of liver RNA*

The results presented in table 26 indicate that the proportions in which

TABLE 26. Effect of liver 65° RNA (pH 8.3) on the ratio of incorporation of different amino acids in the cell-free system of *E. coli*.

RNA fraction	Met	Leu	Asp	Phe
Endogenous	83 (1)	241 (3)	77 (1)	47 (0 6)
65° RNA (pH 8.3)	243 (1)	2000 (11)	447 (23)	812 (4.8)

The reaction mixture contained the components as described in table 6.
Incubations were performed in the presence of L-[Me-^{14}C]-methionine (4 nmoles; 34 mCi/mmole), DL-[l-^{14}C]-leucine (8 nmoles; 34 mCi/mmole) and L-[^{14}C]-aspartic acid (4 nmoles; uniformly labelled; 34 mCi/mmole). The data are given as absolute amounts of measured counts. Values between brackets indicate relative incorporations.

various amino acids are incorporated in the presence of liver RNA are strongly different from the proportions observed in the absence of RNA. One can conclude that in the complete system, incorporation is directed by the exogenous messenger RNA. These observations suggest that there is an inter-relationship similar to lens RNA preparations (table 13).

6 3.9 *Effect of liver RNA on the poly U directed phenylalanine incorporation*

Addition of liver RNA to the incubation mixture in the presence of poly U had a strong stimulatory effect on the phenylalanine incorporation (table 27). In contrast to these results, it was found that poly U had an inhibitory rather than a stimulatory effect on valine and alanine incorporation directed by rat liver RNA (table 28).

Again the effects described here are very similar to those obtained with calf lens RNA (compare tables 14 and 15).

TABLE 27. The stimulatory effect of combined addition of poly U and liver RNA on phenylalanine incorporation.

Addition	Amino acid incorporation counts/min/mg protein	Relative stimulation
None	51	—
None, deproteinized at zero time	30	—
Poly U (60 µg)	6,413	126–fold
Poly U (60 µg) and liver 65° RNA (pH 8 3; 200 µg)	10,083	196–fold
Poly U (60 µg) and liver polysomal RNA (170 µg)	9,780	192–fold
Poly U (60 µg) and liver microsomal (RNA; 200 µg)	7,546	188–fold
Liver 65° RNA (pH 8 3; 200 µg)	443	9–fold
Liver polysomal RNA (170 µg)	98	2–fold
Liver microsomal RNA (200 µg)	103	2–fold

The reaction mixture contained the components as described in table 6.
Incubations were performed in the presence of 4.5 µmoles magnesium acetate, 8 nmoles unlabelled L-phenylalanine and 0 087 nmoles L-[^{14}C]-phenylalanine (uniformly labelled; 504 mCi/mmole). Samples were counted in a thin window gas flow counter.

TABLE 28 The stimulatory effect of combined addition of poly U and liver RNA on valine and alanine incorporation

Addition	Amino acid incorporation counts/min/mg protein	Relative stimulation
None	154	—
None, deproteinized at zero time	84	—
Liver 65° RNA (pH 8 3, 150 μg)	1650	10 1–fold
Liver 65° RNA (pH 8 3, 150 μg) and poly U (60 μg)	1456	9 5–fold
Liver polysomal RNA (170 μg)	334	2 2–fold
Liver polysomal RNA (170 μg) and poly U (60 μg)	307	2 0–fold
Poly U (60 μg)	167	—

The reaction mixture contained the components as described in table 6
Incubations were performed in the presence of 3 5 μmoles magnesium acetate, 20 nmoles DL-[^{14}C]-valine (uniformly labelled, 10 mCi/mmoles) and 20 nmoles DL-[^{14}C]-alanine (uniformly labelled, 10 mCi/mmole) Samples were counted in a liquid scintillation spectrometer

TABLE 29 Effect of rat liver RNA on amino acid incorporation in a cell-free system prepared from rat liver

Addition	Amino acid incorporation counts/minute		Relative stimulation
	Preincubated	Non-preincubated	
None	232	1180	5 6–fold
None, deproteinized at zero time	209	209	—
Minus supernatant (105,000g)	63	108	—
39° RNA (pH 7 6, 150 μg)	276	1517	7 3–fold
Polysomal RNA (150 μg)	238	1432	6 8–fold
65° RNA (pH 8 3, 150 μg)	265	1235	6 0–fold
tRNA rat liver (50 μg)	211	1272	6 0–fold

The cell-free system was prepared according to Bloemendal et al (1966)
Incubations were performed in the presence of 100 μg liver polysomes, 100 μl of 105,000g supernatant, 2 μmoles magnesium acetate, 4 nmoles DL-[l-^{14}C]-leucine (specific activity 34 mCi/mmole) and 8 nmoles unlabelled L-leucine (final volume 0 25 ml) Preincubation was carried out for 30 minutes at 37° C in the absence of RNA and ^{14}C-amino acids Incubations were performed for 30 minutes at 37° C. Samples were counted in a liquid scintillation spectrometer

6.3.10 *Effect of liver RNA on the amino acid incorporation in the homologous cell-free system*

Addition of liver RNA to the homologous cell-free system had no stimulatory effect on the amino acid incorporation in the preincubated system (table 29). On the other hand, addition of RNA to the non-preincubated system had a slight stimulatory effect. These results are comparable to those obtained with lens RNA and can be interpreted in an identical way (chapter 8).

6.3.11 *Sucrose density gradient analysis of liver RNA*

Liver polysomal RNA preparations yielded a sedimentation pattern which was comparable to the pattern shown for lens polysomal RNA (figure 30).

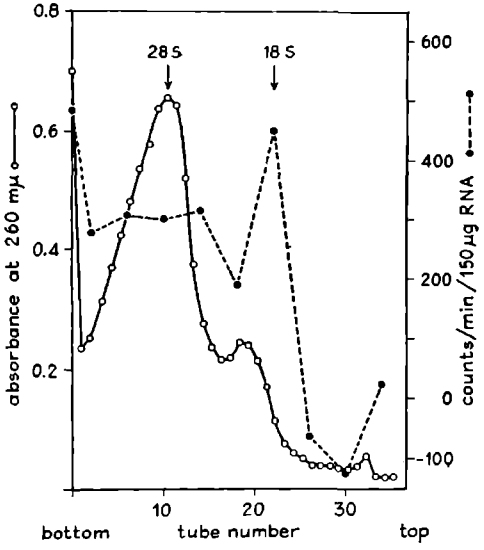

FIGURE 30. Sedimentation pattern of RNA from rat liver polysomes and ability of RNA fractions to stimulate amino acid incorporation into protein.

The reaction mixture contained the components as described in table 6. Incubations were performed in the presence of 8 nmoles DL-[l-^{14}C]-leucine (specific activity 34 mCi/mmole) and 2 nmoles unlabelled L-leucine.
The samples were counted in a thin window gas flow counter.
Incorporated radioactivity in the absence of added RNA has been subtracted from all experimental values.
o———o: absorbance at 260 mμ.
●- - - -●: specific activity (counts/min/200 μg RNA).

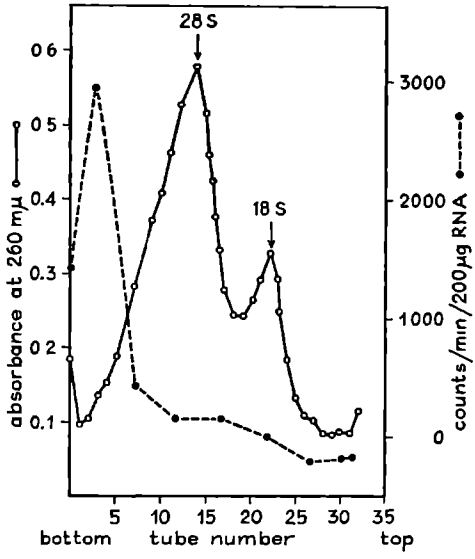

FIGURE 31. Sedimentation pattern of liver microsomal RNA and ability of RNA fractions to stimulate amino acid incorporation into protein

The reaction mixture contained the components as described in table 6. Incubations were performed in the presence of 10 nmoles DL-[l-^{14}C]-leucine (specific activity 55 2 mCi/mmole), 1.5 nmole L-[^{14}C]-aspartic acid (uniformly labelled, specific activity 217 mCi/mmole), 0.5 nmole L-[^{14}C]-phenylalanine (uniformly labelled; specific activity 504 mCi/mmole), 10 nmoles unlabelled L-phenylalanine, 10 nmoles unlabelled L-aspartic acid and 10 nmoles unlabelled L-leucine The samples were counted in a liquid scintillation spectrometer
In all figures incorporation in the absence of added RNA has been subtracted
o———o· absorbance at 260 mμ
●- - - -● · specific activity (counts/min/200 μg RNA)

In the case of liver polysomal RNA, however, the 28S peak was significantly higher than the 18S peak, and low molecular weight material on the top of the gradient was virtually absent. Assays of messenger activity of the fractions obtained after sucrose density gradient centrifugation, revealed that there was a high stimulating activity in the 10S region (figure 30). RNA fractions near to and at the bottom of the tube had a specific activity which was of the same order of magnitude as the specific activity of RNA fractions in the 10S region (figure 30). Fractions near to the top of the gradient inhibited the incorporation possibly because of tRNA and oligonucleotides (Di Girolamo et al., 1964; Aaronson et al., 1966).

RNA isolated from the microsomal fra
pattern nearly identical to the pattern of
above (figure 31). Again virtually no ma
present at the top of the gradient. Assa
microsomal RNA fractions from the sucro
which the highest specific activity was fc
tube. Fractions near the top of the gradier
incorporation. It should be mentioned, hov
tion in the distribution of the messenger a
the highest specific messenger activity was
this shift of specific activity was caused
RNA remains to be explained.

Sucrose density gradient centrifugation
and at pH 8.3 revealed a sedimentation pat

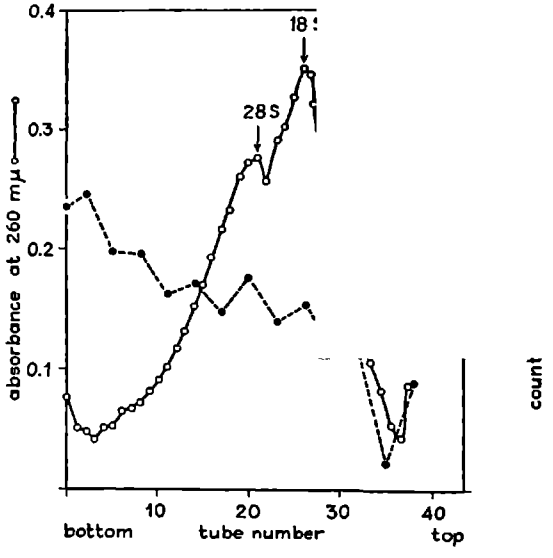

FIGURE 32. Sedimentation pattern of liver 65° RNA (pH 8.3) and ability of RNA fractions to stimulate amino acid incorporation into protein.

The reaction mixture contained the components as described in table 6. Incubations were performed in the presence of 8 nmoles DL-[l-^{14}C]-leucine (specific activity 34 mCi/mmole) and 2 nmoles unlabelled L-leucine. Samples were counted in a thin window gas flow counter.
In all figures incorporation in the absence of added RNA has been subtracted.
o———o: absorbance at 260 mμ.
●– – – –●: specific activity (counts/min/150 μg RNA).

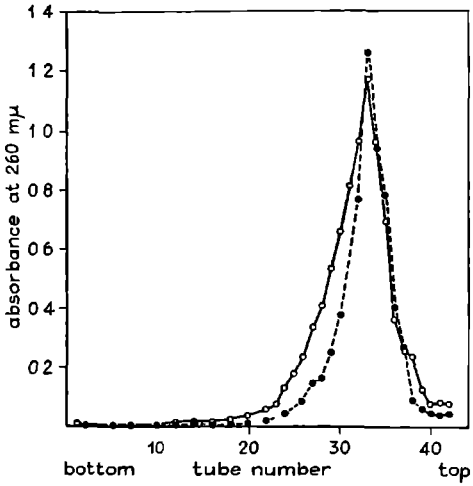

FIGURE 33 Sedimentation pattern of *E coli* ribosomes in the presence and absence of rat liver RNA

E coli ribosomes (350 μg) were incubated (10 min at 0°C) in the presence and absence of rat liver 65° RNA (100 μg) in 0 5 ml Tris-HCl buffer, pH 7 8, containing 60 mM NH_4Cl and 14 mM magnesium acetate After incubation the mixture was layered on a sucrose gradient (8–22%) containing 14 mM magnesium acetate, 60 mM NH_4Cl, buffered to pH 7 8 with 10 mM Tris-HCl and centrifuged for 2 hours at 25,000 rev/min in rotor SW 25 1

●– – – –● Sedimentation pattern of E coli ribosomes incubated in the absence of rat liver 65° RNA (pH 8 3)

o———o Sedimentation pattern of *E coli* ribosomes incubated in the presence of rat liver 65° RNA (pH 8 3)

from the patterns mentioned above However, the pattern was very similar to the pattern obtained from lens RNA extracted under the same conditions The highest amount of material absorbing at 260 mμ was found in the 18S region (figure 32) Measurements of messenger activity of fractions from the sucrose gradient revealed that there was a continuous increase in specific activity towards the bottom of the tube (figure 32) These results are comparable to the results obtained with lens RNA extracted at 65°C (pH 8 3)

The meaning of these results will be discussed in chapter 8

6 3 13 *Binding of liver RNA to purified ribosomes of E coli*

As has been pointed out in chapter 2, the first step in protein synthesis is the binding of messenger RNA to the ribosomes In order to demonstrate

that polysomes are formed after incubation of liver 65° RNA with *E. coli* ribosomes, the incubated mixture was analysed by sucrose density gradient centrifugation. The results (figure 33) indicate that in the denser fractions there is a small shift towards the bottom of the gradient of the material absorbing at 260 mμ.

This result is again an indication of the presence of messenger RNA in our RNA preparations.

6.4 CONCLUSIONS

The messenger activity of corresponding RNA preparations from calf lens and rat liver was of the same order of magnitude.

RNA extracted from polysomes or from the microsomal fraction had the lowest messenger activity. The highest messenger activity was consistently found in RNA preparations extracted at 65° C and alkaline pH (8.3).

The amino acid incorporation directed by exogeneous RNA was dependent on the magnesium concentration. The optimal incorporation for both lens and liver RNA was reached at a magnesium concentration of 14 mM.

The kinetics of amino acid incorporation directed by lens and liver RNA were essentially the same. Maximal incorporation was observed after about 45 minutes of incubation.

The amino acid incorporation was dependent on the amount of RNA added to the cell-free system. The plateau reached depended on the RNA fraction studied.

Heating and rapid cooling had virtually no effect on the messenger activity of RNA preparations both from lens and liver.

With the aid of sucrose density gradient centrifugation a binding of liver 65° RNA (pH 8.3) to *E. coli* ribosomes could be demonstrated.

Re-initiation of protein synthesis by addition of more RNA after 45 minutes of incubation, could not be achieved.

Both lens RNA and liver RNA had a striking stimulatory effect on the poly U directed phenylalanine incorporation. On the other hand, poly U had no effect on the amino acid incorporation directed by liver and lens RNA in cases where no phenylalanine was used.

The ratio of incorporation of different amino acids varied when exogeneous RNA, isolated from either lens or liver, was added to the preincubated cell-free system of *E. coli*.

Addition of lens or liver RNA to the homologous preincubated cell-free system had no stimulatory effect on the amino acid incorporation.

Corresponding RNA preparations from calf lens and from rat liver yielded nearly the same sedimentation profiles, as judged by sucrose density

gradient analysis. Messenger activity was widely distributed throughout the gradient. A slight maximum was usually found in the 10S region but, the highest specific activity was consistently found near to and at the bottom of the tubes.

A better resolution of lens polysomal RNA by chromatography on MAK columns could not be achieved. Differences in specific messenger activity between various fractions of the column eluate could not be demonstrated.

Binding of N-acetylmethionyl-tRNA and methionyl-tRNA to *E. coli* ribosomes in the presence of lens RNA could be demonstrated, whereas an incorporation into protein of N-acetylmethionine from N-acetylmethionyl-tRNA in the cell-free system from lens cells could not.

FURTHER CHARACTERIZATION OF RNA PREPARATIONS WITH MESSENGER ACTIVITY, ISOLATED FROM CALF LENS CELLS AND RAT LIVER

7.1 INTRODUCTION

In the previous chapter the characteristics of isolated RNA preparations with respect to their stimulatory activity on the amino acid incorporation, were described. As pointed out in chapter 3, this was not the only criterion used to characterize our RNA preparations on the presence of messenger RNA. Base analysis and electrophoretic data on polyacrylamide gels are the other criteria used to characterise these RNA preparations. The results of these investigations will be described in this chapter.

7.2 BASE COMPOSITIONS OF RNA PREPARATIONS FROM CALF LENS AND RAT LIVER

7.2.1 *Introduction*

One of the properties frequently used for the characterization of messenger RNA is that the RNA fraction in question should have a base composition which is very similar to homologous DNA.

In the literature several RNA preparations with such homology to DNA are described (Harris, 1964; Henshaw et al., 1965; Tsanev et al., 1966b; Roberts and Quinlivan, 1969). However, the base compositions of these RNA preparations were determined with the aid of the so-called "pulse label" technique. Several objections can be made both to the use of this method, and indeed, to the use of RNA-DNA base homology as a criterion of the presence of messenger RNA.

In our opinion a better criterion for messenger RNA, would be a base composition which differs from those of ribosomal, 5S and transfer RNA. It has to be borne in mind, however, that at present it is impossible to isolate messenger RNA completely free from ribosomal or transfer RNA. Therefore, it can be expected that most messenger RNA preparations have a base composition which to some extent is similar to the base composition of ribosomal RNA.

The base compositions of our isolated RNA preparations are given in

table 30. For comparison, base compositions of ribosomal RNA, homologous calf thymus DNA, and rat liver DNA as reported respectively by Hadjivassi-

TABLE 30. Nucleotide composition of RNA fractions isolated from calf lens cells or from rat liver.

Tissue	RNA fraction	AMP	GMP	CMP	UMP	G-C %
lens	Polysomal RNA	19.7	33.3	29.9	17.1	63
		19.6	34.0	30.2	16.2	64
	0° RNA (pH 7.6)	18.8	34.0	29.3	17.9	63
		19.8	33.1	30.0	17.1	63
	39° RNA (pH 7.6)	21.0	33.2	29.5	16.3	63
		19.5	33.7	30.0	17.8	63
	39° RNA (pH 8.3)	20.6	33.9	27.5	18.0	61
		20.4	34.2	27.4	18.0	62
	65° RNA (pH 8.3)	21.5	31.5	28.2	18.8	60
		21.5	31.8	28.5	18.2	60
liver	Polysomal RNA	18.5	34.6	29.0	17.9	64
		18.7	35.3	26.7	17.3	64
	39° RNA (pH 7.6)	21.0	33.2	29.5	16.3	63
		19.5	33.7	30.0	17.8	63
	39° RNA (pH 7.6) *	17.8	33.3	31.3	17.5	65
		17.7	33.0	31.8	17.5	65
	65° RNA (pH 8.3)	22.5	30.8	28.2	18.8	59
		22.4	30.3	28.4	18.9	59
	microsomal RNA	19.0	32.9	30.8	17.3	64
		17.7	33.3	31.7	17.2	65
	tRNA+	19.0	33.3	27.7	19.8	61
		19.5	33.0	28.3	19.2	61
	0° RNA (pH 7.6) **	21.2	31.3	27.2	20.3	59
		22.0	32.4	26.1	19.5	59
	39° RNA (pH 7.6) **	23.7	35.3	21.0	20.0	56
		23.6	35.3	20.8	20.3	56
liver	ribosomal RNA (Hadjivassiliou and Brawerman, 1965)	18.0	32.8	31.2	18.0	64
		dAMP	dGMP	dCMP	dTMP	
liver	DNA (rat) (Wyatt, 1951)	28.6	21.4	21.5	28.4	43
thymus	DNA (calf) (Chargaff, 1963)	27.4	22.4	20.7	29.5	43

Values are expressed as moles per 100 moles total nucleotides.

* Extracted from regenerating rat liver, 20hr after partial hepatectomy.

** Extracted from purified nuclei.

\+ Odd-bases not determined.

liou and Brawerman, (1965); Chargaff (1963) and Wyatt (1951) have also been included.

It can be concluded that our RNA preparations exhibit a base composition which is dissimilar to that of DNA. Moreover, the base compositions of RNA preparations isolated in the same way from calf lens and rat liver are nearly identical and do not deviate much from those of ribosomal RNA. However, RNA preparations extracted from purified nuclei or RNA extracted at 65° C (pH 8.3) from both calf lens and rat liver, have a G-C content which is significantly lower than the G-C content of the other RNA preparations. The G-C content of 65° RNA (pH 8.3) was sometimes even lower (57%).

This indicates that these RNA species contain a high proportion of RNA with base compositions significantly different from ribosomal RNA. These results are in accordance with the results recently obtained by other investigators on RNA preparations extracted from rat liver. (Mizuno et al., 1967, 1968).

It should be noted that RNA preparations with the highest stimulatory activity of amino acid incorporation (cf. chapter 6) also have a base composition which is widely different from the base composition of rRNA.

7.2.2 *Base composition of RNA fractions obtained after sucrose density gradient centrifugation*

From the results described above it can be concluded that most of our RNA preparations have a ribosomal RNA-like base composition. However, the results given in chapter 6 show that the stimulatory effects on amino acid incorporation of RNA fractions obtained after sucrose density gradient centrifugation, are not all of the same order of magnitude. As already mentioned, we looked for RNA preparations with a base composition different from ribosomal RNA. For this reason we tried to correlate these messenger activities with base compositions deviating from the base composition of ribosomal RNA.

The results obtained from base analysis of RNA fractions after sucrose density gradient centrifugation, are given in table 31.

From these results it can be concluded that there is no marked difference between the G-C content of different RNA fractions isolated from the sucrose gradients. Only the pellet obtained after sucrose density gradient centrifugation of lens 65° RNA (pH 8.3) had a G-C content which was significantly lower. The C content of lens polysomal RNA fractions decreased towards the top of the gradient. These results may be related to the lower G content of 18S ribosomal RNA compared to 28S ribosomal RNA. A comparable result could be observed for the G-content of polysomal RNA from rat liver.

TABLE 31 Nucleotide composition of RNA fractions obtained after sucrose density gradient centrifugation

RNA fraction	Sucrose density gradient fraction	AMP	GMP	CMP	UMP	G-C %
lens polysomal RNA (figure 21)	tubes 1–10	18 5	30 9	33 2	17 4	64
		18 8	31 5	33 0	16 7	65
	tubes 10–19	17 9	35 0	31 9	15 2	67
		18 7	34 1	31 5	15 7	66
	tubes 19–28	18 6	32 0	30 9	18 5	63
		19 1	32 1	30 3	18 5	62
	tubes 28–36	19 0	35 5	28 2	17 3	64
		19 7	35 4	28 5	17 4	63
liver polysomal RNA (figure 30)	pellet	19 9	36 0	28 9	15 2	65
		20 3	37 1	27 4	15 2	65
	tubes 1–10	19 3	35 2	27 5	18 1	63
		20 5	34 8	27 0	17 7	62
	tubes 10–19	20 6	33 3	28 7	17 4	62
		20 6	33 9	28 6	16 9	62
	tubes 19–28	20 2	34 3	29 6	15 9	64
		20 5	33 9	28 9	16 7	63
	tubes 28–36	22 3	32 6	27 9	17 1	61
		21 7	32 1	28 7	17 6	61
liver microsomal RNA (figure 31)	pellet	20 6	33 7	28 0	17 7	62
		20 3	34 8	27 7	17 3	62
lens 65° RNA (pH 8 3) (figure 23)	pellet	21 6	30 8	27 1	20 5	58
		20 6	32 0	26 2	19 5	58

Values are expressed as moles per 100 moles total nucleotides

7 3 POLYACRYLAMIDE GEL ELECTROPHORESIS OF RNA PREPARATIONS ISOLATED FROM CALF LENS AND RAT LIVER

7 3 1 *Introduction*

With the introduction of polyacrylamide gel electrophoresis there was the possibility of separating high molecular weight RNA with a resolution which was hitherto impossible (Bishop et al , 1967, Loening, 1967, Peacock et al , 1967, Grossbach and Weinstein, 1968, Egyházi et al , 1968; Ringborg et al , 1968) In addition highly detailed analysis of the synthesis and breakdown of ribosomal RNA became possible (Gould, 1966a, 1966b, Gould et al , 1966, McPhie et al , 1966, Weinberg et al , 1967)

In this section the results obtained from polyacrylamide gel electrophoresis using a variety of RNA preparations extracted from calf lens and rat liver, are presented The results will be compared with other procedures used for

the fractionation of RNA preparations. In addition some special characteristics of these RNA preparations will be described.

7.3.2 *Electropherograms of RNA preparations isolated from calf lens and rat liver*

Polyacrylamide gel electrophoresis of some RNA preparations isolated from calf lens and rat liver, revealed very heterogeneous patterns of well defined bands with different mobilities (Figure 34a and 34b). Apparently the resolving power of polyacrylamide gel electrophoresis is much higher than that of density gradient centrifugation (figures 21–23) or that of an analytical ultracentrifuge (figure 35). It is striking that the patterns from RNA preparations isolated by the same procedure from calf lens and rat liver are absolutely identical.

The major bands of 28S and 18S ribosomal RNA can easily be located. In addition, there are several minor bands above, between and below the ribosomal RNA bands. Recently, some of these bands have also been obser-

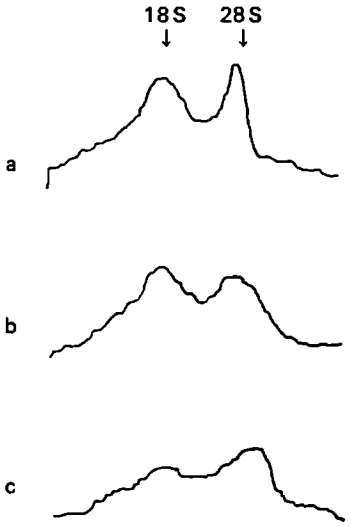

FIGURE 35. Ultraviolet recording of the sedimentation pattern of lens and liver RNA. RNA was dissolved in 0.01 M sodium acetate (pH 5.1) and 100 mM sodium chloride in a final concentration of 40 µg per ml.
Recordings at 50,740 rev/min, 6 min after reaching maximum speed.
a. polysomal RNA lens
b. 39° RNA lens (pH 7.6)
c. polysomal RNA liver.

ved by other investigators after electrophoresis of RNA preparations isolated from rat liver, kidney, brain or Novikoff Hepatoma, respectively (Peacock et al., 1967; King and Fitschen, 1968; Prestayko et al., 1968; Moriyama et al., 1969). The significance of these results will be discussed in the next chapter.

7.3.3 *Electropherograms of RNA fractions obtained after sucrose density gradient centrifugation*

Electrophoresis on polyacrylamide gels of RNA fractions obtained after sucrose density gradient centrifugation, revealed that in each fraction several well separated bands could be observed, which were not seen on the sucrose gradients (figures 36 and 37). It could also be observed that there was a close relationship between the electrophoretic mobility of the RNA fraction in question and their sedimentation value (figures 21–23). Similar patterns were obtained from the sucrose density gradient fractions from all other RNA preparations. Electrophoresis of RNA from the bottom of the gradient showed that this fraction, in general, was disperse and contained RNA species with a sedimentation value higher than 28S.

7.3.4 *Effect of heat and urea treatments of lens and liver RNA studied with polyacrylamide gel electrophoresis*

Several cases of aggregate formation between different RNA species are described in the literature (Asano, 1965; Kirby, 1965; Marcot-Queiroz and Monier, 1965b; Hayes et al., 1966a; 1966b). These aggregates were mainly found at high salt concentrations ($>$ 0.3 M NaCl).

We tried to find whether complexes were present in our RNA preparations. For this reason the RNA preparations were treated with 6 M urea followed by electrophoresis in polyacrylamide gels containing 6 M urea. From figure 38 it can be concluded that this treatment had a dramatic effect on the pattern (migration velocity) of lens and rat liver RNA. Unfortunately, after this treatment a large amount of RNA (presumably RNA with a sedimentation value higher than 18S) could not penetrate the polyacrylamide gel. This may be the result of a conformational change of the RNA molecule, possibly from a coiled to a stretched arrangement. Another explanation may be a change in the nature of the polyacrylamide gel matrix due to polymerization in the presence of 6M urea. The molecular sieve properties of such polyacrylamide gels resemble 3.5% gels prepared in the absence of 6M urea (Peacock and Dingman, 1967).

An additional observation was that after 6M urea treatment the number and intensity of the minor bands above the 4S RNA region increased.

Presumably this result is an indication for the presence of complexes in our RNA preparations. The nature and function of these molecules are, however, still obscure.

Polyacrylamide gel electrophoresis of RNA fractions obtained after sucrose density gradient centrifugation in the presence of 6M urea resulted in patterns which were comparable to those obtained after centrifugation in the absence of urea (figures 36 and 37). However, in the former case the RNA bands were somewhat more diffuse, and the A260 absorbance pattern was shifted into the direction of the 18S peak.

Another possible means of splitting RNA complexes is heating and rapid cooling (Wilkinson, 1965). The results of such treatment are given in figure 39. In contrast to the results obtained after 6M urea treatment a more intensive accumulation of material below the 18S rRNA band was observed. The effect of heating 28S rRNA is more pronounced than for 18S rRNA. A similar shift in the electrophoretic pattern, though less pronounced, was also observed after heating at lower temperatures (37°C) (see also Peacock and Dingman, 1967). If heating in the presence of urea, and heating before urea treatment was performed, it had the same effect as had treatment with urea alone (figure 40). These results may indicate that RNA molecules are *fragmented or that complexes are dissociated due to the heat treatment.* (cf. Warner et al., 1967). Fragmentation due to RNase action seems unlikely for three reasons. 1. Heating in the presence of an RNase inhibitor (bentonite) has no effect on the electrophoretic pattern. 2. RNA incubated at 0°C has the same pattern as non-incubated RNA. 3. Treatment with RNase has a dramatic effect on the electrophoretic pattern, and only material with a higher electrophoretic mobility than 4S RNA can be observed.

The observed non-enzymatic change of ribosomal RNA after heat treatment has also been observed by many other investigators (Hall and Doty, 1959; Petermann et al., 1963; Brown and Doty, 1968; Tsanev, 1965; Applebaum et al., 1966; Dabeva and Tsanev, 1966; Midgley, 1966; Bramwell et al., 1967; Venkov et al., 1967; Tsanev and Russev, 1968). These results as well as our electrophoretic data suggest that rRNA might consist of subunits. Moreover, the following observations support such a conclusion. The chain length of rRNA's from *E. coli* (Midgley et al., 1967; Nichols et al., 1967), wheat germ (Lane, 1965; Diemer et al., 1966), rat liver and Ehrlich ascitus tumor cells (Tsanev and Russev, 1968) were found to be much shorter than could be expected on the basis of their sedimentation behaviour. These results may also explain why, in contrast to the expectation, the highest A260 absorbance peak after phenol extraction at high temperatures is shifted from the 28S to the 18S region (see figures 23 and 32).

7.4 CONCLUSIONS

Base analysis of RNA preparations isolated from calf lens and rat liver, revealed that their base composition in general did not deviate much from the base composition of ribosomal RNA. The RNA fractions which had the highest stimulatory activity on amino acid incorporation into protein also had the lowest G-C content.

A correlation between the highest messenger activity and G-C content deviating from that of rRNA, in RNA fractions obtained after sucrose density gradient centrifugation of lens polysomal RNA and lens 39° RNA (pH 7.6) could not be demonstrated.

Polyacrylamide gel electrophoresis of RNA preparations isolated from calf lens and rat liver revealed a very heterogeneous pattern of well-defined bands with different mobilities. The electrophoretic mobilities of the various RNA fractions obtained after sucrose density gradient centrifugation were in accordance with their sedimentation behaviour. In addition these RNA fractions, with the exception of the most dense fractions, gave well-separated bands which could not be observed spectrophotometrically on the sucrose gradient.

Heating and urea treatment of RNA had a dramatic effect on its mobility, and also resulted in an accumulation of material with a lower molecular weight. It is proposed that these latter effects indicate a subunit composition of ribosomal RNA.

GENERAL DISCUSSION

In the present investigation a comparison was made between the messenger activities of RNA preparations from calf lens and rat liver. The results obtained indicate not only that in all RNA preparations messenger activity could be detected, but also that there is a striking similarity in the levels of messenger activity provided the RNA preparations from different sources are isolated according to the same procedure. These observations will be discussed in more detail in this chapter, and a general comparison will be made between our results and those reported by other investigators.

Stimulation of amino acid incorporation into protein

All RNA preparations isolated by the described procedures from calf lens and rat liver, had a stimulatory effect on amino acid incorporation into protein in the cell-free system prepared from *E. coli*. These findings can be explained in two different ways:

a. stimulation is the result of the protection and consequent translation of endogenous *E. coli* messenger RNA as proposed by Drach and Lingrel (1966b) (see also Howells and Wyatt, 1969);

b. stimulation is the result of the translation of exogenous lens and/or liver messenger RNA.

Drach and Lingrel (1966b) isolated polysomal RNA from reticulocytes and added it to the cell-free system of *E. coli*. They then mapped the peptides which had been synthesized in this *in vitro* system, and claimed that the protein synthesized resembled *E. coli* protein rather than reticulocyte globin. They suggested that the added RNA afforded some kind of protection for the endogeneous messenger RNA against RNase degradation.

The present results on the effects of calf lens and rat liver RNA on the poly U directed phenylalanine incorporation lend support to this assumption. In contrast to expectation, a stimulation of phenylalanine incorporation was noted, which was independent of the type of RNA added (table 14) (cf. Aaronson et al., 1966; Maxwell et al., 1966). These results also suggest that at a high magnesium concentration (18 mM), poly U is bound to the

ribosomes in preference to the lens or liver messenger. This effect was not observed at a lower magnesium concentration (14 mM) suggesting that poly U had virtually no effect at this lower concentration on the incorporation directed by lens or liver RNA. It is therefore quite possible that the "non-informational" RNA present in our preparations already protects the lens and liver messenger against degradation. This would result in addition of poly U having no stimulatory effect.

Addition of RNase inhibitor prepared from rat liver according to Gribnau et al. (1969) had no stimulatory effect on the amino acid incorporation. This may indicate that the stimulation is not due to a protection against RNase degradation. However, it cannot be excluded that the RNase inhibitor has some specificity.

Results which favour the assumption that it is the exogenous messenger that is being translated are the following:

a. the stimulatory effect of the RNA preparations on the amino acid incorporation into protein was strongly dependent on the RNA fraction in question;

b. RNA preparations from different sources, but isolated according to the same procedure had nearly the same stimulatory effect on the incorporation and finally,

c. the highest stimulatory activity was always found in RNA preparations isolated from crude or purified nuclei. The base composition of these preparations deviated from that of ribosomal RNA.

The results mentioned above are in agreement with the results obtained by other investigators with RNA preparations isolated from mammalian tissues (see tables 1, 2 and 3 and Georgiev, 1967; Hadjiolov, 1967), and provide evidence for the presence of messenger RNA in the isolated RNA preparations.

Additional evidence for the presence of messenger RNA in our RNA preparations are the following observations:

a. the relative incorporations of four different amino acids in the presence of lens or liver RNA differed significantly from the endogenous incorporation of the test system;

b. incubation of *E. coli* ribosomes in the presence of rat liver RNA (65°C, pH 8.3) followed by gradient centrifugation, resulted in a slight shift of the sedimentation pattern towards the bottom of the tube. This shift probably indicates that the major part of liver messenger RNA has only a few ribosomal binding sites. Limited binding sites for *E. coli* ribosomes have also been

demonstrated for large mRNA molecules from viral origin (Voorma, 1965; Dahlberg and Haselkorn, 1966; 1967a);

c. the magnesium requirement for optimal amino acid incorporation depends on which messenger is present. This observation may be related to a difference of initiation mechanism required to start translation of bacterial and mammalian messenger RNA. In the former case protein synthesis is known to start with N-formylmethionine (see chapter 2.4) while the initiation mechanism in the latter case has still to be elucidated. From the observation that the N-terminus of α-crystallin appeared to be N-acetyl-met-asp-ile-ala- (Hoenders et al. 1968b) it seemed plausible to suppose that protein synthesis in the lens starts with N-acetylmethionine. However, there is no indication for the presence of a mechanism which acetylates methionyl-tRNA in bacterial systems.

It might well be, therefore, that the amino acid incorporation directed by mammalian RNA in the cell-free system of *E. coli* starts in a non-enzymatic manner (compare with 2.5.2, Reinecke et al., 1968). Another possibility is that chain initiation does start enzymatically with the recognition of the chain initiation codon AUG. As there is no proof that protein synthesis in the lens and liver starts with N-blocked methionine, it is not excluded that AUG codons present in non-terminal positions of lens and liver mRNA function as chain initiation signals. However, it is also not excluded that these initiation signals are formed as the result of the overlapping of two adjacent triplets (e.g. GUA UGG). Observations which favour this latter assumption are those of N-formylmethionine incorporation directed by plant virus RNA (Hoogendam et al., 1968).

Re-initiation of protein synthesis after the addition of extra portions of lens or liver RNA to the incubation mixture could not be achieved. This observation may be the result of a general decrease in the incorporating ability of the test system, or the absence of chain termination signals due to the degradation of mRNA during incubation. If this is true the ribosomes remain bound to the mRNA and consequently no free ribosomes are made available (compare 2.6).

Addition of lens or liver RNA to the pre-incubated cell-free system prepared from rat liver or calf lens also had no enhancing effect on the amino acid incorporation. This failure of the homologous cell-free system to translate additional messenger RNA may be the consequence of there being no active initiation mechanism.

Fractionation of RNA preparations

Sucrose density gradient analysis of RNA preparations with messenger

activity both from lens and liver, demonstrated their heterogeneity. Although messenger activity was distributed throughout the gradient the highest specific stimulatory effect on amino acid incorporation into protein was obtained with RNA fractions from the 10S region and especially from fractions near to, and at the bottom of the tube. There are several results described in the literature which support these observations (see also table 2). Stewart and Papaconstantinou (1967a, 1967b), working with cultured lenses, found the highest incorporation of ^{3}H-uridine in RNA fractions at the bottom of their sucrose gradients. Willems et al. (1968) were able to isolate an RNA fraction from the nucleoplasm of Hela cells, which was very heterogeneous in nature and which had a base composition which was different from ribosomal RNA. Large heterogeneous RNA species (30 to 80 S) have also been isolated from rat liver nuclei and rat liver polysomes (Henshaw, 1968; Penmann et al. 1968; Schütz et al., 1968). It is proposed that such high molecular weight RNA is transported to the cytoplasm in the form of an RNA protein complex (Parsons et al., 1968; Samarina et al., 1966; 1968; Henshaw et al., 1965; Perry and Kelly, 1966; 1968; McConkey and Hopkins, 1965; Spirin, 1966; Infante and Nemer, 1968 Nemer, 1967; Kafatos, 1968 and see also 6.2.10).

Our results together with these observations may imply that in mammalian cells there is, in addition to messenger RNA of low molecular weight (10S, monocistronic), messenger RNA of high molecular weight ($>$ 28S, polycistronic).

The high resolving power of polyacrylamide gel electrophoresis enabled a considerable heterogeneity of the fractions isolated from the sucrose gradients to be demonstrated. On the other hand, the remarkable similarity of patterns of RNA from such different tissues as lens and liver, is very striking. Several minor bands above, between and below the major rRNA bands could also be observed. The number and appearance of the minor bands between 28S and 18S rRNA are virtually identical. Similar minor bands have also been observed in RNA preparations from *Chironomus tentans* (Grossbach and Weinstein, 1968) rat brain or liver (King and Fitschen 1968; Dingman and Peacock, 1968; Peacock and Dingman, 1968), but the nature and function of these bands still has to be determined. Some of them may simply represent products of enzymatic degradation while others may be specific classes of RNA not previously resolved by conventional procedures. The concentration of some of the minor bands appears to be much lower than those from ribosomal RNA and would, therefore, be in the range expected for messenger RNA. However, the similarity between electropherograms is so great that the identification of any particular band

as mRNA is not possible. This may mean that the set of mRNA's present in these cells hardly differs from tissue to tissue or indeed between the rat and calf.

On the other hand, it is possible that no mRNA could be demonstrated in the electrophoretic studies as the concentration of such species was to low. Fractionation of RNA with the aid of polyacrylamide gel electrophoresis on a preparative scale might provide an answer to these questions.

Electrophoresis of RNA from the more dense regions of sucrose gradients revealed that this fraction was disperse and appeared to contain molecular weight species with sedimentation values higher than 28S. A possible explanation for these findings has been given by Grossbach and Weinstein (1968) who observed that if the gel pore size was within a critical range, RNA would concentrate on the gel surface, but if the voltage gradient was maintained, part of this RNA could migrate into the gel producing a diffuse smear. The existence of well-defined mRNA species in the bottom fractions of the sucrose gradients can therefore not be excluded.

Although the presence of messenger activity in our RNA preparations has been demonstrated, at least according to the generally accepted criteria, the *in vitro* synthesis of a specific lens or liver protein has still to be demonstrated.

In this thesis a study was made of messenger activity in RNA preparations isolated from calf lens and rat liver. The main criterion used to detect messenger activity was the ability of the RNA fraction in question to stimulate amino acid incorporation into protein in a cell-free system prepared from *E. coli* B.

All RNA preparations stimulated the amino acid incorporation into protein, but the extent of stimulation varied considerably from fraction to fraction. Both for calf lens and rat liver RNA the highest specific messenger activity was found in RNA preparations isolated at rather high temperatures (65°C) and at alkaline pH (8.3). High stimulatory activity could also be demonstrated in RNA preparations from purified rat liver nuclei. The highest stimulatory activity was found in RNA fractions, the base composition of which deviated most from the base composition (G-C content) of ribosomal RNA. In this connection it should be mentioned that RNA isolated at 65°C from the crude nuclear lens pellet had a somewhat higher content of ribosomal RNA than RNA extracted at 65°C from rat liver. This may explain the somewhat higher messenger activity and lower G-C content of the latter.

The kinetics and magnesium dependence of amino acid incorporation were very similar for lens and for liver RNA. The optimal magnesium requirement for amino acid incorporation directed by lens or liver RNA differed slightly from the corresponding magnesium requirement of the endogenous mRNA directed amino acid incorporation. Amino acid incorporation was strongly dependent on the amount of RNA added to the incubation mixture. The amount of RNA necessary for optimal incorporation depended on the type of RNA.

Data obtained with four different radioactive amino acids demonstrated that their ratio of incorporation when directed by RNA fractions either from calf lens or rat liver, was significantly different from the endogenous incorporation of the *E. coli* test system. When RNA preparations were added to the preincubated homologous cell-free system, there was no stimulatory effect on the amino acid incorporation. RNA fractions both from calf lens and rat liver strongly stimulated the poly U directed phenylalanine incorporation. The reverse effect could not be demonstrated.

Heating (65°C, 15 min) and rapid cooling had virtually no stimulatory effect on the lens or liver RNA directed amino acid incorporation. In contrast

heating of these RNA preparations resulted in a completely different electrophoretic pattern in polyacrylamide gels. A sharp difference in electrophoretic behaviour on polyacrylamide gels could also be observed after heating both in the presence and absence of urea and after treatment with urea alone.

Sucrose density gradient analysis of RNA fractions with messenger activity both from calf lens and rat liver demonstrated their heterogeneity. Although some activity was distributed throughout the gradient the highest stimulatory effect on the amino acid incorporation was obtained with RNA fractions in the 10S region and near to and at the bottom of the tube. Chromatography of lens polysomal RNA on MAK-columns did not result in a concentration of the messenger activity in a well-defined fraction.

Due to the high resolving power of polyacrylamide gel electrophoresis a considerable heterogeneity of the fractions isolated from the sucrose gradients could be demonstrated. On the other hand, the great similarity of both the sucrose density gradient profiles, and the electrophoretic patterns of RNA fractions isolated in the same way from such different tissues as calf lens and rat liver, was very striking.

The electrophoretic pattern of RNA from the most dense fractions of the sucrose gradients was disperse. These lowest fractions correspond to sedimentation values higher than 28S. Preliminary results indicate that in the presence of lens RNA, N-acetylmethionyl-tRNA is bound to ribosomes. However, more searching experiments must be performed before it can be concluded that the synthesis *in vitro* of lens proteins can be started with N-acetylmethionine. In addition, it has to be determined whether the biosynthetic product has an amino acid sequence corresponding to one of the sequences of lens or liver proteins.

SAMENVATTING

In dit proefschrift is een onderzoek beschreven over matrijs-activiteiten in RNA-fracties, geïsoleerd uit kalfslens en rattelever. Als voornaamste criterium om deze activiteit in de op verschillende wijzen geïsoleerde RNA-fracties aan te tonen, gold het vermogen om de aminozuurinbouw in het cel-vrije systeem van *E coli* B te stimuleren.

Alle verkregen RNA-fracties stimuleerden de aminozuurincorporatie in eiwit, maar de mate van stimulering was sterk afhankelijk van de wijze waarop de RNA fractie was geïsoleerd. Zowel voor kalfslens- als rattelever-RNA werd de hoogste activiteit gevonden in fracties welke bij relatief hoge temperaturen (65°C) en bij alkalische pH (8,3) waren geïsoleerd. Hoge matrijsactiviteiten konden ook worden aangetoond in RNA-fracties welke uit gezuiverde rattelever-kernen waren verkregen.
RNA-fracties met de hoogste matrijs-activiteiten hadden tevens een basensamenstelling (G-C gehalte) welke het sterkst van die van ribosomale RNA afweek. In verband hiermede dient te worden opgemerkt, dat RNA geïsoleerd bij 65°C uit de ruwe lenskernen-fractie een hoger gehalte aan ribosomale RNA heeft dan RNA geëxtraheerd bij 65°C uit rattelever.
Dit laatste gegeven is tevens een verklaring voor de iets hogere matrijs-activiteit en lager G-C gehalte van laatstgenoemde RNA-fractie.

De tijds- en magnesium-ionenconcentratie-afhankelijkheid, van de aminozuurincorporatie gestimuleerd door lens- en lever-RNA, waren vrijwel identiek. De maximale magnesium ionenconcentratie van de door lens- of lever-RNA gestimuleerde aminozuurincorporatie verschilde slechts weinig van de door endogeen matrijs-RNA gestimuleerde aminozuurincorporatie. De aminozuurincorporatie was verder sterk afhankelijk van de hoeveelheid RNA welke aan het incorporatiesysteem werd toegevoegd. Bovendien hing de hoeveelheid RNA, welke voor een optimale incorporatie nodig was, nauw met de aard van de toegevoegde RNA samen.

De verhoudingen, waarin vier verschillende radioactieve aminozuren bij aan- en af-wezigheid van lens- en lever-RNA in eiwit werden ingebouwd, verschilden sterk van elkaar. Toevoeging van RNA-fracties aan het gepreincubeerde homologe cel-vrije systeem had geen stimulerend effect op de aminozuurincorporatie. RNA-fracties welke zowel uit de kalflens als rattelever waren geïsoleerd, vertoonden een sterk stimulerend effect op de door

polyuridylzuur gestimuleerde phenylalanine incorporatie Het omgekeerde effect kon echter niet worden aangetoond

Verwarming (65°C, 15 min) en snelle afkoeling van lens- en lever-RNA had vrijwel geen effect op de respectievelijke matrijsactiviteiten Daarentegen werd na verwarming en afkoeling van deze RNA-fracties een geheel ander polyacrylamidegel-electroforesepatroon verkregen Een ander electroforetisch gedrag werd ook waargenomen wanneer deze RNA-fracties in aan en afwezigheid van ureum werden verhit of alleen met ureum werden behandeld

Door middel van suikergradient-analysen kon een grote heterogeniteit van de verschillende RNA-fracties worden aangetoond Hoewel matrijsactiviteiten in vrijwel alle fracties van de suikergradient kon worden aangetoond, werd de hoogste specifieke matrijs-activiteit vrijwel altijd gevonden in RNA-fracties met een sedimentatiewaarde van ongeveer 10S en in RNA-fracties aanwezig in de bodemfracties van de gradient Fractionering van lens polysomale RNA met behulp van gemethyleerde albumine kieselguhrkolommen resulteerde niet in een concentrering van matrijs-activiteiten

Als gevolg van het grote oplossend vermogen van polyacrylamidegels kon een grote heterogeniteit in de RNA-fracties, welke na suikergradient centrifugering werden verkregen, worden aangetoond Daarentegen was de grote overeenkomst tussen de suikergradientpatronen en electroferogrammen van, op analoge wijze geisoleerde RNA-fracties, uit zulk een verschillende weefsels als de kalflens en rattelever, zeer opvallend Het electroforesepatroon van RNA geisoleerd uit de bodemfracties van de suikergradienten was diffuus. Deze fracties correspondeerden met sedimentatiewaarden welke groter waren dan 28S. Inleidende experimenten toonden aan, dat N-acetylmethionyl-tRNA bij aanwezigheid van lens-RNA aan lens-ribosomen wordt gebonden

Verder experimenten dienen echter te worden uitgevoerd alvorens geconcludeerd kan worden, dat de synthese van lenseiwitten met behulp van N-acetylmethionine start Bovendien dient te worden aangetoond, dat het biosynthetisch product een aminozuurvolgorde heeft, welke met de volgorde van respectievelijk lens- en lever-eiwitten correspondeert

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aa	–	amino acid
A	–	adenine or adenosine monophosphate
ADP	–	adenosine-5'-diphosphate
ala	–	alanine
AMP	–	adenosine-5'-monophosphate
AMV	–	Alfalfa Mosaic Virus
asp	–	aspartic acid
arg	–	arginine
ATP	–	adenosine-5' -triphosphate
BMV	–	Bromegrass Mosaic Virus
C	–	cytosine or cytosine monophosphate
DNA	–	deoxyribonucleic acid
DNase	–	deoxyribonuclease
E. coli	–	*Escherichia coli*
EDTA	–	ethylenediamine tetraacetate
fm	–	formylmethionine
F-met	–	formylmethionine
fTHF	–	formyltetrahydrofolate
G	–	guanine or guanosine monophosphate
GMP-PCP	–	5'-guanylyl-methylene-diphosphate
GDP	–	guanosine-5'-diphosphate
gly	–	glycine
glu	–	glutamic acid
GTP	–	guanosine-5'-triphosphate
GTPase	–	guanosine triphosphatase
ile	–	isoleucine
leu	–	leucine
MAK	–	methylated albumin kieselguhr
met	–	methionine
NDS	–	naphtalene-I-5-disulphonic acid
PCA	–	perchloric acid
PEP	–	phosphoenol pyruvate
phe	–	phenylalanine
Pi	–	orthophosphate
PK	–	pyruvate kinase
Poly U	–	polyuridylic acid
POPOP	–	4,4-bis(2–5-phenyl) oxazolyl benzene
PPi	–	pyrophosphate
PPO	–	2,5- diphenyloxazol
PVS	–	polyvinyl sulphate
RNA	–	ribonucleic acid
mRNA	–	messenger ribonucleic acid
rRNA	–	ribosomal ribonucleic acid
tRNA	–	transfer ribonucleic acid

RNase	–	ribonuclease
RNP	–	ribonucleoprotein
SDS	–	sodium dodecyl sulphate
ser	–	serine
STNV	–	Satellite of Tobacco Necrosis Virus
T	–	thymidine or thymidine monophosphate
TCA	–	trichloroacetic acid
THFA	–	tetrahydrofolic acid
thr	–	threonine
TMV	–	Tobacco Mosaic Virus
TNV	–	Tobacco Necrosis Virus
Tris	–	tris (hydroxymethyl) aminomethane
TYMV	–	Turnip Yellow Mosaic Virus
tyr	–	tyrosine
U	–	uracil or uridine monophosphate
val	–	valine

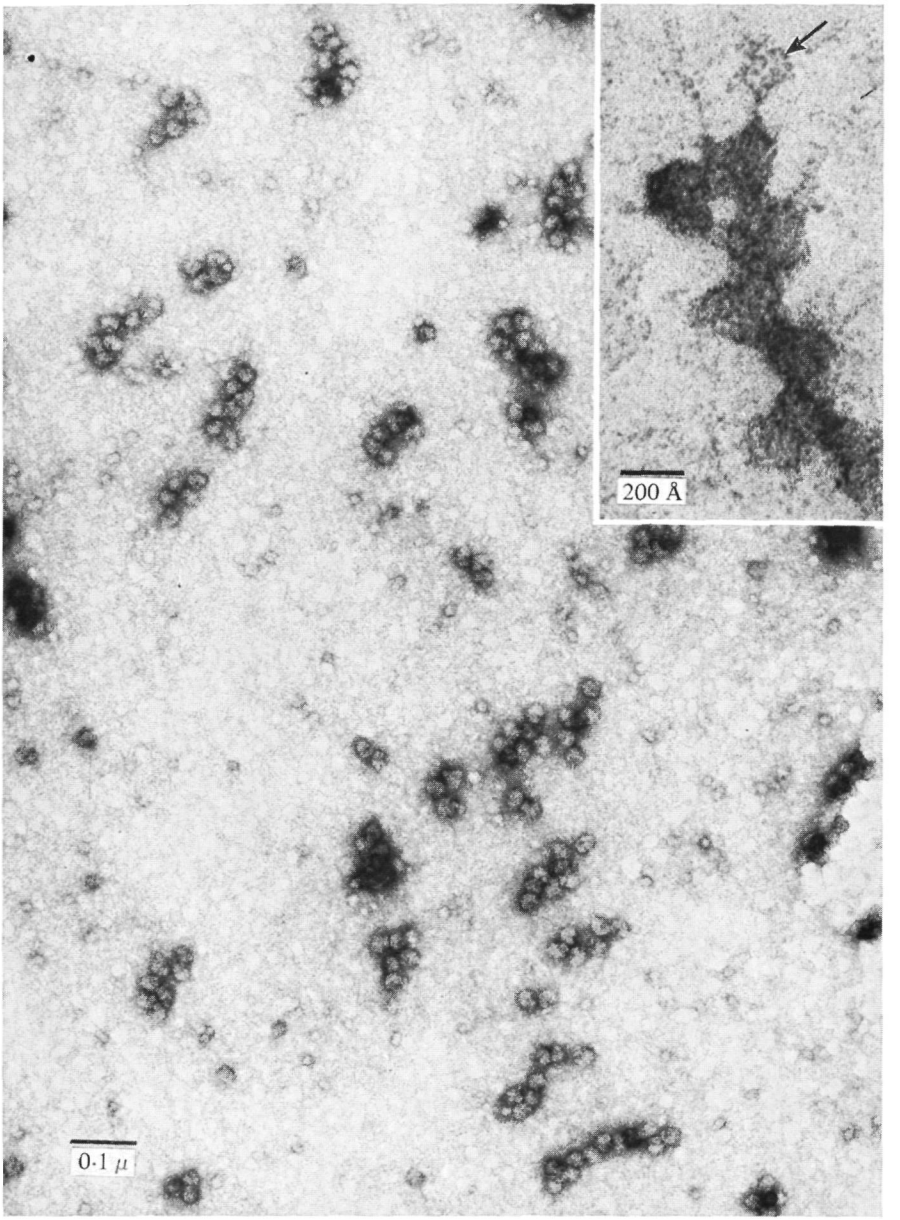

FIGURE 5. Electron micrograph of lens polysomes isolated as indicated, spread on carbon film and stained with 1% uranylacetate. Polysomes consisting of nine monomers can easily be observed. (Reproduced from E. L. Benedetti, A. Zweers and H. Bloemendal, 1968, with permission).

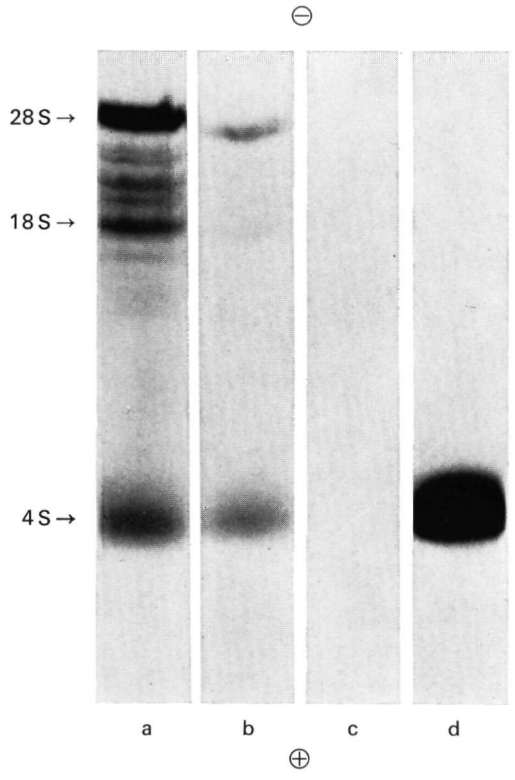

FIGURE 6. Polyacrylamide gel electrophoresis of RNA fractions obtained during the isolation and purification of tRNA from rat liver.

a. RNA fraction obtained after the first phenol extraction.
b. soluble RNA fraction obtained after addition of IM NaCl.
c. RNA fraction not retained by DEAE-Sephadex A-50 after loading the column.
d. RNA fraction eluted from the DEAE-Sephadex A-50 column at an NaCl concentration range from 0.4 – 0.7 M.

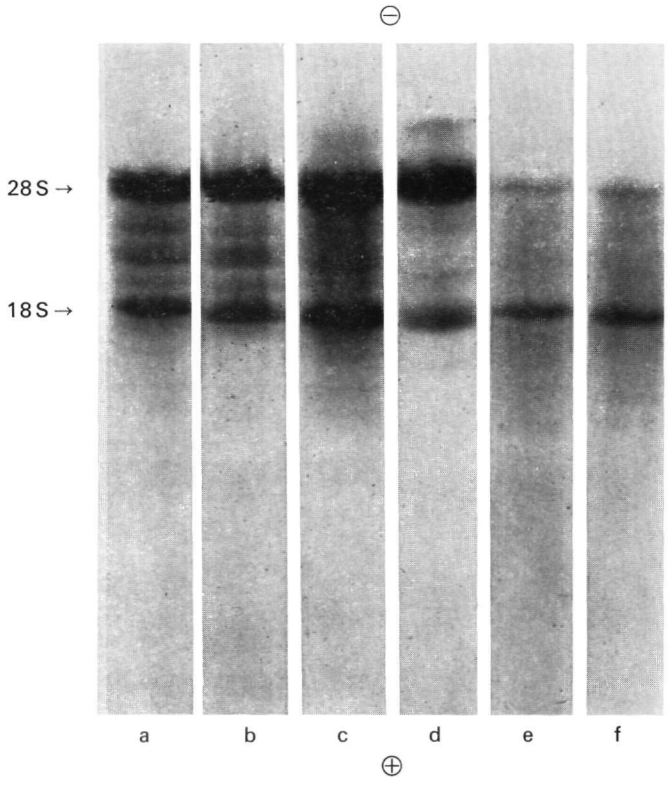

FIGURE 34a. Polyacrylamide gel electrophoresis of RNA fractions from calf lens and from rat liver.

a. polysomal RNA lens
b. polysomal RNA liver
c. 39° RNA lens (pH 7.6)
d. 39° RNA liver (pH 7.6)
e. 65° RNA lens (pH 8.3)
f. 65° RNA liver (pH 8.3)

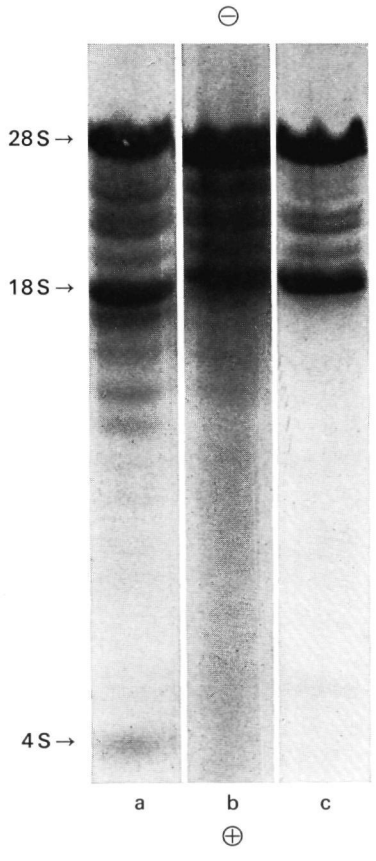

FIGURE 34b. Polyacrylamide gel electrophoresis of RNA fractions from calf lens and rat liver.
a. polysomal RNA lens
b. 0° RNA lens (pH 7.6)
c. microsomal RNA liver

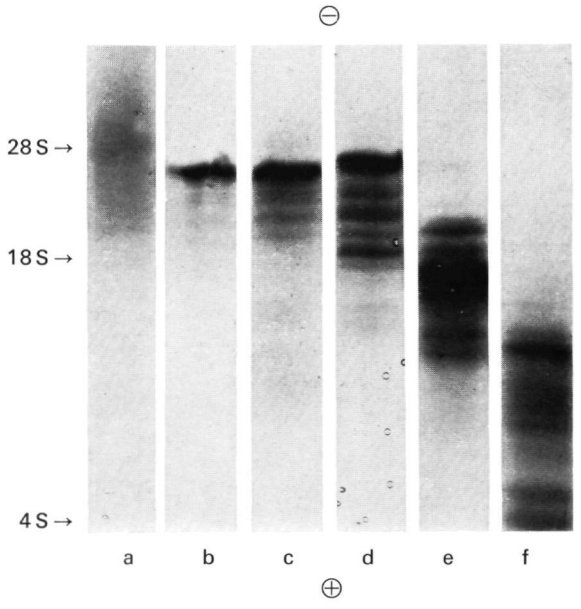

FIGURE 36. Polyacrylamide gel electrophoresis of lens polysomal RNA isolated from separated sucrose density gradient fractions. The samples applied were pooled fractions from the sucrose gradient as shown in figure 21.

a. bottom-fraction 7
b. fraction 7–13
c. fraction 13–19
d. fraction 19–25
e. fraction 25–31
f. fraction 31–37

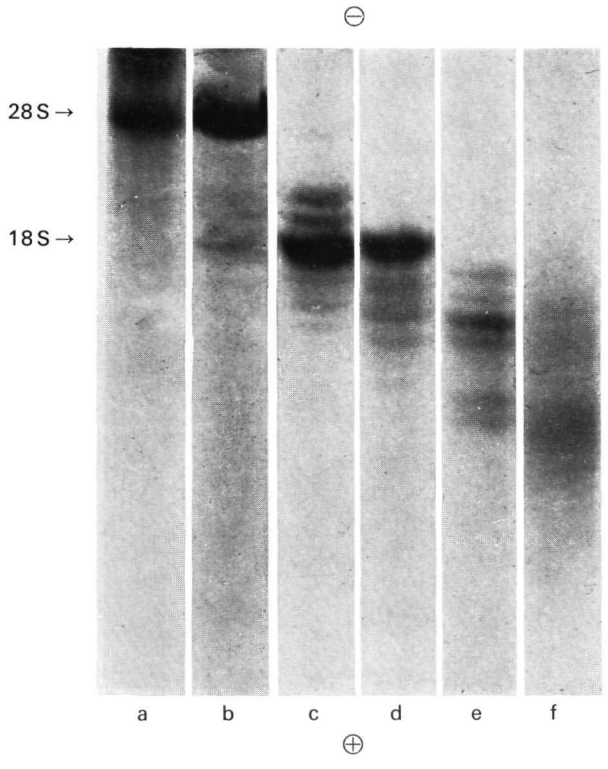

FIGURE 37. Polyacrylamide gel electrophoresis of lens 39° RNA isolated from separated sucrose density gradient fractions. The samples applied were pooled fractions from the sucrose gradient as shown in figure 22.

a. bottom – fraction 9
b. fraction 9–16
c. fraction 16–21
d. fraction 21–24
e. fraction 24–28
f. fraction 28–31

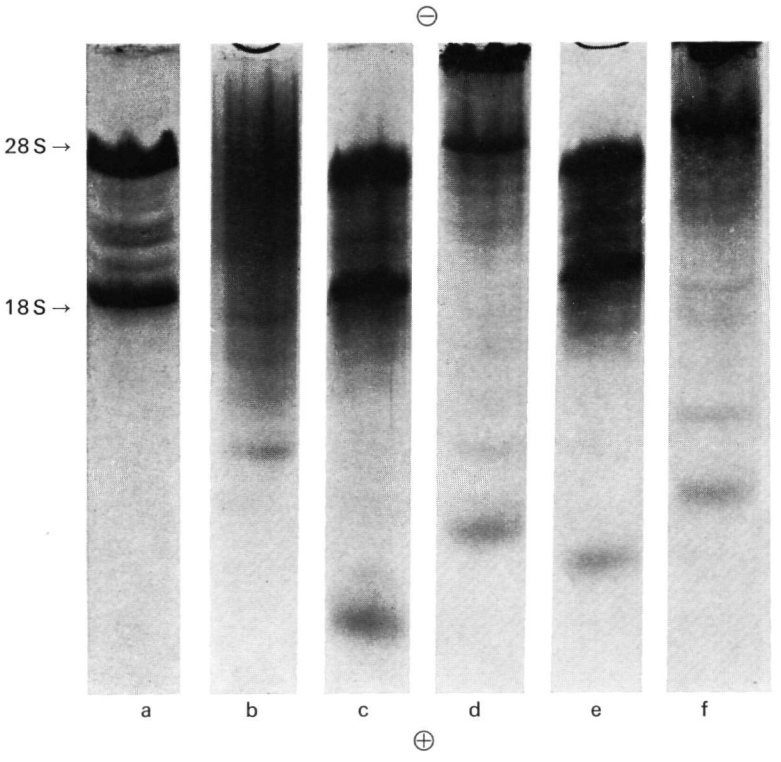

FIGURE 38. Effect of heating and cooling on electrophoretic mobility of different RNA preparations from calf lens and rat liver.

a. lens polysomal RNA
b. lens polysomal RNA (heated)
c. liver polysomal RNA
d. liver polysomal RNA (heated)
e. 0° RNA lens (pH 7.6)
f. 0° RNA lens (pH 7.6), (heated)

Heating was carried out in 40 mM Tris buffer, pH 7.2, containing 20 mM sodium acetate and 3 mM EDTA for 15 min at 70° C.

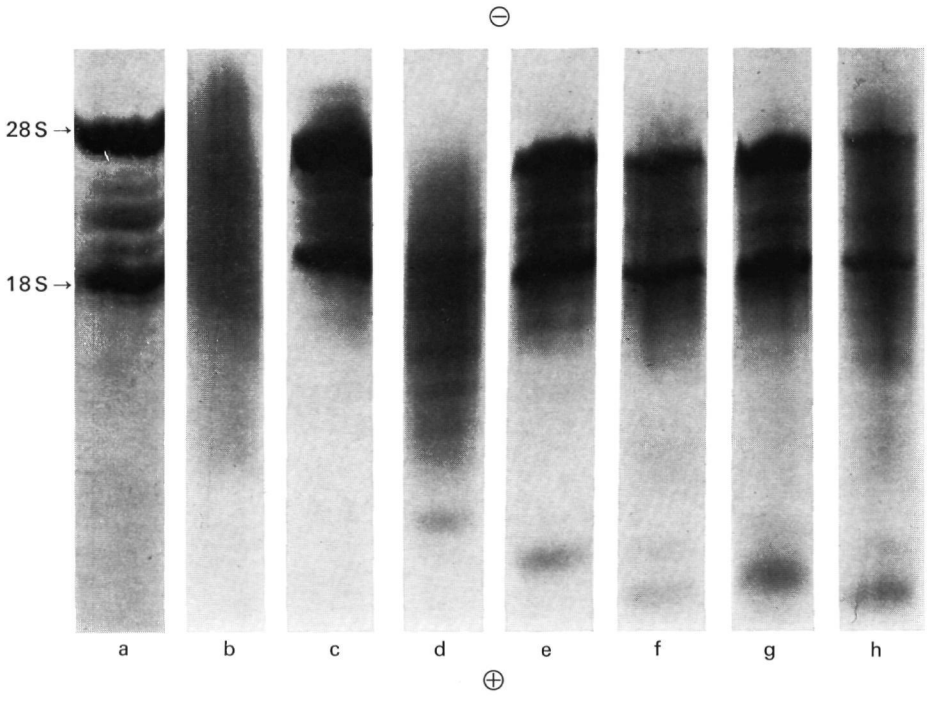

FIGURE 39. Effect of 6 M urea treatment on RNA preparations isolated from calf lens and rat liver.

Electrophoresis was performed as described in 4.

a. lens polysomal RNA
b. lens polysomal RNA + 6 M urea
c. 0° RNA lens (pH 7.6)
d. 0° RNA lens (pH 7.6) + 6 M urea
e. 39° RNA lens (pH 7.6)
f. 39° RNA lens (pH 7.6) + 6 M urea

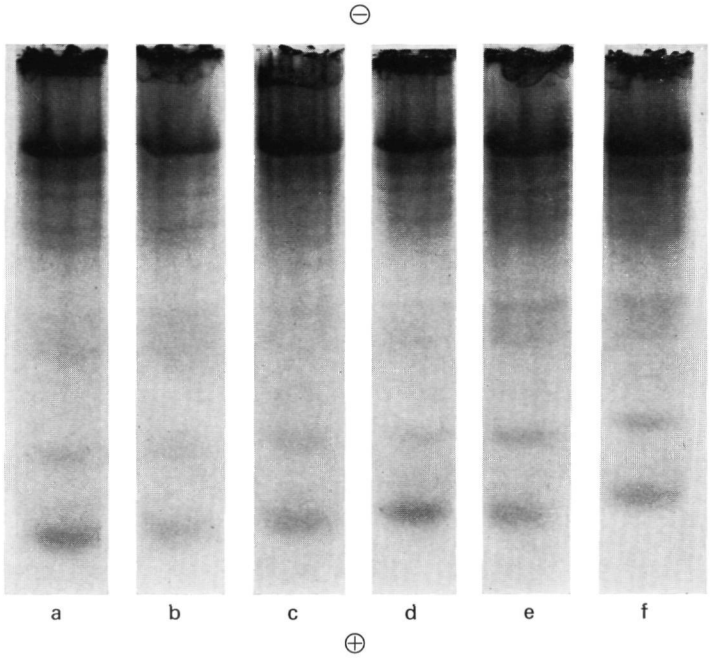

FIGURE 40. Effect of heating and 6 M urea treatment on RNA preparations isolated from calf lens.

a. 0° RNA lens + 6 M urea
b. 0° RNA lens (heated) + 6 M urea*
c. 0° RNA lens + 6 M urea (heated)
d. 39° RNA lens (pH 7.6) + 6 M urea
e. 39° RNA lens (pH 7.6), (heated) + 6 M urea*
f. 39° RNA lens + 6 M urea (heated)

Heating was carried out in 40 mM Tris buffer, pH 7.2, containing 20 mM sodium acetate and 3 mM EDTA for 15 min at 70° C.

* Heating was carried out before urea treatment was performed.

STELLINGEN

I

Het door Papaconstantinou en Julku opgestelde model voor de regulatie van de ribosomale RNA synthese in de vertebrate lens, is aanvechtbaar.

J Papaconstantinou en E M Julku (1968) J Cell Physiol *72*, 161

II

De conclusie van Aguilera en medewerkers, dat polyuridylzuur door γ-bestraling van waterige oplossingen van uridylzuur wordt gevormd, is onvoldoende gemotiveerd

A Aguilera, E Colombara, R Jiménez en J Toha (1965) Biochim Biophys Acta *95*, 569

III

De verschillen tussen de hemocyanines van Arthropoda en Mollusca suggereren dat beide molecuulsoorten onafhankelijk van elkaar zijn ontstaan

W N Konings (1969) Proefschrift Groningen

IV

Dat in ongekiemde stuifmeelkorrels van *Tradescantia paludosa* grotere polysomen voorkomen dan in gekiemde korrels, is een conclusie welke in strijd is met de experimentele gegevens

J P Mascarenhas en E Bell (1969) Biochim Biophys. Acta *179*, 199.

V

Het katalytisch effect van phosphatidyl-ethanolamine, op de vorming van 2,3-dialkylacroleïnen uit aldehyden, kan worden verklaard door aan te nemen dat een geprotoneerde Schiffse base als intermediair optreedt

H H O Schmid en T Takahashi (1968) Hoppe-Seyler's Z Physiol Chem *349*, 1673

VI

De eisen, welke momenteel gesteld worden aan de lozing van afvalwater op onze meren en rivieren, zijn vastgelegd in de Hinderwet, Keuren van Waterschappen en de Wet Verontreiniging Oppervlaktewateren Een spoedige en drastische wijziging van deze eisen is van zeer groot belang voor het behoud van het bestaande biologische evenwicht

VII

Bij moleculairgewichtsbepalingen volgens het principe van Archibald, waarbij de concentratie en de afgeleide onafhankelijk van elkaar worden gemeten, wordt niet voldoende rekening gehouden met het feit dat laagmoleculaire verontreinigingen de uitkomst sterk onbetrouwbaar maken.

W. J. Archibald (1947) J. Phys. and Colloid Chem. *51*, 1204.
W. S. Bont en W. L. Van Es (1967) Anal. Chim. Acta *38*, 147.
K. de Groot, J. C. M. Reynen en H. J. Hoenders (1969) Anal. Biochem. in press.

VIII

Voor een meer vruchtbare en efficiënte beoefening van de research aan de nederlandse universiteiten dient de research zoveel mogelijk van het onderwijs te worden gescheiden. Bovendien dient het autonome karakter van de bestaande instituten te worden opgeheven.

IX

Het is niet onwaarschijnlijk dat het verouderingsproces aan de hand van de verandering in entropie primair bestudeerd kan worden.

X

Structuurbepaling van ribosomen met behulp van de electronenmicroscoop is nog weinig zinvol.

N. Nanninga (1968) Proc. Natl. Acad. Sci. U.S. *61*, 614.
V. I. Bruskov en N. A. Kiselev (1968) J. Mol. Biol. *37*, 367.
A. Worcel, D. S. Goldman en J. B. Sachs (1968) Proc. Natl. Acad. Sci. U.S. *61*, 122.

XI

De bewering van Kroeger, dat de $Na^- - K^+$ balans in de celkern regulerend werkt op de vorming van het „puff-patroon" tijdens de ontwikkeling van Diptera larven, is onwaarschijnlijk en bovendien ongefundeerd.

H. Kroeger (1966) Exp. Cell Res. *41*, 64.

XII

Het kweken van de mens in de „reageerbuis" dient onder direct toezicht van een wereld omvattende organisatie komen te staan.

R. N. H. Konings

Nijmegen, 24 juni 1969